



**The Metabolic Basis  
of  
Inherited Disease**





# The Metabolic Basis of Inherited Disease

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## Preface

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The astonishing growth of human biochemical genetics during the past few years has created a need for a critical and comprehensive account of those heritable disorders of metabolism for which an appreciable body of knowledge now exists. This book is an effort to meet this need by presenting the pertinent clinical biochemical and genetic information concerning those metabolic anomalies which have been grouped under Garrod's engaging term—the inborn errors of metabolism. Recent studies have clarified the nature of a number of these diseases; new disclosures have brought others within the group; and indeed new syndromes have been uncovered.

In order to secure authoritative presentations the editors enlisted the collaboration of investigators actively engaged in the intensive study of specific heritable diseases. Each contributor was given ample freedom to organize his own material as he might see fit within the broad framework of a general format. He was encouraged to include sufficient normal biochemical and metabolic material in the relevant fields in order to provide a firm foundation for a detailed exposition of the nature of the defect at the heart of the disease or condition under scrutiny. He was also urged to indicate areas where further work is needed and to hazard some speculations from existing data. In this manner we attempted to achieve a stimulating and contemporary interpretation of each disease against a background of normal human intermediary metabolism and physiology.

An entirely satisfactory solution to the problem of the selection of topics and organization was not possible and many arbitrary decisions had to be made. For example, we omitted discussions of several diseases which might logically have been included, such as the heritable disorders of connective tissue recently well reviewed by Victor A. McKusick. The problem of classification was resolved largely on a basis of convenience. Most disorders were categorized according to superficial biochemical resemblance but some eluded this scheme and seemed best grouped by organ system of chief involvement. This has been done with some misgiving and with full awareness that a genetically determined



disorder cannot logically be considered limited to one system of the body

Each chapter has been critically reviewed by each of the editors and a number have been reviewed by additional readers. Among the latter we are particularly indebted to Drs William Beck, John Bertles, Roscoe Brady, Charles Burnett, Evan Calkins, Allen Crocker, Bernard Davis, Leslie J. DeGroot, Frank Engel, Jordi Folch Pi, Lamont W. Gaston, John Graham, Mahlon Hoagland, V. M. Ingram, Richard Portwood, Lloyd H. Smith, Jr., Robert Stempfel, Eberhard Trams, and T. Franklin Williams. Many of the sections on genetics were reviewed by Dr. H. Eldon Sutton of the Department of Human Genetics of the Medical School of the University of Michigan. The efforts at excellence put forth by all authors and their tolerant acceptance of suggested changes in their manuscripts have made the tasks of the editors pleasant indeed.

Our secretaries, Miss Elizabeth Callahan and Mrs. Lenora Wells, have given us devoted and invaluable assistance. Much of the illustrative material was skillfully drawn by Mrs. Edith Tagrin of the Medical Art Department of the Massachusetts General Hospital. The continued advice, support, and encouragement of Dr. Walter Bauer, Dr. James Howard Means, Dr. Eugene A. Stead, Dr. Philip Handler, and Dr. Robert Berliner have been of immeasurable help to the three of us.

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## Part One

### Introduction

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## Chapter 1

# Inherited Variation and Metabolic Abnormality

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*The Editors*

Man is endowed with great genetic diversity. While distinct stocks may be recognized by the anthropologist, genetically homogeneous strains of man do not exist. With the singular exception of monozygotic twins, each member of the human race has a unique inheritance. Inevitably, individual variation is an obvious feature of man.

Individual variation is partly hereditary and partly environmental. The environmental factors which influence the expression of the hereditary traits are of great variety, perhaps greater than those to which any other species is exposed. And yet, man's individuality varies only within relatively narrow confines. His similarities include such chemical factors as composition of tissues and directions and rates of metabolic processes.

Deviations from the norms of chemical constancy may occur for a variety of reasons. These deviations may be simply extremes of distributions of traits. Most deviations represent consequences of acquired dysfunction. A significant number represent heritable disorders attributable to mutant genes. These examples are important out of all proportion to their numerical incidence because of the lessons they teach of the nature of metabolic events and their dependence upon hereditary control.

Not all mutations embarrass the host in dealing with his environment. The benignity of some mutations has obscured their presence and possibly distorted our view of the constancy of metabolic processes. Other mutations undoubtedly cause alterations in metabolism which are unrecognized because they are incompatible with viability. Between these extremes lies a growing number of examples of deviant metabolism whose diverse clinical expressions belie a close kinship in origin. These conditions all may root in a common soil represented by the etiologic formulation: altered genetic information → abnormal structure of a given protein in the host → an effect upon the chemical activity of that protein.



To be sure, for many of the conditions included in this volume this formulation is only a framework for speculation at present. In a few instances, however, information of such specificity concerning causal mechanism had been acquired that the term *molecular disease* has seemed an appropriate designation [1]. The promise of continuing enlightenment in this area is bright, a fortunate state of affairs which is a tribute to a century of outstanding contributions in the converging fields of clinical medicine, biochemistry and genetics.

The subsequent chapters are concerned with the present state of advance in the biochemistry of inheritance and with many specific instances of genetically altered metabolism. For the reader uncertain of the terrain which lies ahead, a brief pause for perspective is offered in the following few pages into both historical development and the general features of control of intermediary metabolism most applicable to the inherited diseases. Other more extensive reviews devoted to this same purpose are available elsewhere [2-9].

## HISTORICAL CONSIDERATIONS

### CONCEPT OF INBORN ERRORS OF METABOLISM (GARROD)

Shortly before the turn of the present century Sir Archibald Garrod began his studies on alcaptonuria which were to culminate in his classic Croonian Lectures of 1908 [10] and in his monograph on *Inborn Errors of Metabolism*, which appeared in 1909 and again in 1923 [11]. From his observations on alcaptonuria, cystinuria, albinism and pentosuria, Garrod developed the concept that certain diseases of lifelong duration arise because an enzyme governing a single metabolic step is reduced in activity or missing altogether. Garrod viewed the accumulation of homogentisic acid in alcaptonuria as evidence that this substance was a normal metabolite in the dissimilation of tyrosine and correctly attributed its accumulation to a failure of oxidation of homogentisic acid. A half century later Garrod's hypothesis was proved by demonstration of the absence of homogentisic acid oxidase activity in the liver of a patient with alcaptonuria [12].

Similarly, the failure of pigment formation in the skin in albinism, the appearance of pentose in the urine in essential pentosuria and the excretion of large amounts of cystine in the urine in cystinuria were viewed by Garrod as the results of blocks in normal metabolic pathways. He attributed the first instance to failure of melanin formation and the latter two to excretion of metabolites accumulating proximal to the block in metabolism. His interpretation of the defect in cystinuria has required modification, but the correctness of his remaining hypotheses has been supported by subsequent studies.

The fundamental discovery of Mendel concerning the inheritance of plant characters [13] had only just been resurrected and given prominent consideration at the time when Garrod was most active. The transmission of a single character as a governing agent for a single enzyme and accordingly, as one step in intermediary metabolism is a thesis which seems implicit in Garrod's writings, but it was never stated as such. The term *gene* was not introduced by Johannsen [14] until 3 years after Garrod's Croonian Lectures and nearly 40 years more were to pass before Beadle [15] clearly formulated the 'one gene-one enzyme' concept (see below).

Garrod's work was almost ignored by geneticists for a generation. Indeed Beadle [8] was led to remark that an examination of the major textbooks of genetics in use in 1940 failed to reveal any mention of alcaptonuria. Time has corrected this oversight, the contributions of Garrod now occupy a deservedly prominent place in genetics and have had a marked effect on conceptual thinking in clinical medicine as well.

## THE CHEMICAL TRANSLATION OF GENETIC INFLUENCE

### *Plant Pigments*

Studies on the inheritance of flower colors initiated by a contemporary of Garrod probably were the first to demonstrate genetic influence at the molecular level. These investigations begun by Wheldale [16-18] and extended by Scott Moncrieff [19], Lawrence [20] and many others come close to marking the beginnings of biochemical genetics. Several types of changes at single loci in the structure of the principle water soluble plant pigments, the anthocyanins, are inheritable and these substitutions cause great differences in pigment color [21].

At present it is thought that a gene governing a given chemical step in the production of plant pigments exerts its influence by controlling the synthesis or activity of an enzyme specific for that step [22-23]. The early intuition of Wheldale that inheritable differences in flower color correspond to specific differences in pigment molecules was clearly a forerunner of the concept that a single chemical reaction may be under the control of a specific genetic locus.

### *One Gene-One Enzyme*

The concept mentioned above attained clear definition in the 'one gene-one enzyme' principle first clearly stated by Beadle [15]. This formulation of such fundamental importance to understanding of the inherited disease emerged gradually from studies of eye color in the fruit fly *Drosophila* begun 30 years ago by Beadle and Ephrussi and co-workers [24-25]. It received extensive support from the classic studies of

Beadle and Tatum on induced mutants of *Neurospora crassa* in which the acquisitions of single essential growth factors were traced to single chemical reactions each dependent upon a different enzyme [8, 26]

The one gene-one enzyme concept which Beadle developed from the experiments has been well expressed by Tatum [9] as follows

- 1 All biochemical processes in all organisms are under genic control
- 2 These biochemical processes are reducible into series of individual stepwise reactions
- 3 Each biochemical reaction is under the ultimate control of a different single gene
- 4 Mutation of a single gene results only in an alteration in the ability of the cell to carry out a single primary chemical reaction

The one gene-one enzyme concept has recently been expressed in more sophisticated terms [27] embodying modifications that have developed since its inception. These include a realization that the one gene-one enzyme concept does not require a one enzyme-one gene corollary, and the replacement of the 'gene' by progressively smaller units of ultimate genetic control. Significant evidence contrary to the general theory has not been introduced, however, and many subsequent studies have supported it.

### *Inherited Diseases as Errors in Protein Synthesis*

The manner in which genetic information may govern the activity of an enzyme through altering its structure has been brought into sharp focus in the last decade by a series of brilliant studies on inheritable differences in structure of a nonenzymatic protein—the globin portion of the hemoglobin molecule. This work, initiated by Pauling and his colleagues [1] is comprehensively reviewed in Chaps. 2 and 34 and need be only briefly summarized here.

It was first observed by Pauling, Itano, Singer, and Wells [1] that sickle-cell hemoglobin moves differently in an electrical field than does normal hemoglobin—a finding which suggested to them that there might be a difference in amino acid composition between the globin moieties of normal and sickle cell hemoglobin to explain their apparent difference in charge. This inference was subsequently borne out by the elegant studies of Ingram [cf. 28]. Employing a 'finger printing' technique for peptide identification, he demonstrated that the structural difference between the two hemoglobins lay in only one of the many peptide fragments produced by trypsin hydrolysis. Further analysis indicated this difference to be the substitution of one residue of valine for one of glutamic acid. Thus the disadvantageous physical properties of sickle-cell hemoglobin result from a change in only one of several hundred amino acid residue. A similar single substitution has now also been found to account for the different properties of several other hemoglobins.

These exciting studies with hemoglobin have made it easy to visualize

how changes in the activity of an enzyme may be introduced under genetic control, since doubtless that activity is dependent upon many physical properties of the protein molecule which in turn are likely to be radically affected by minor structural changes

### ENZYMES AND METABOLIC REACTIONS

A detailed inquiry into the characteristics of enzymes and of mechanisms of enzymatic action is beyond the scope of this book [29] But since many disorders to be considered involve enzymatic derangements a few considerations are in order

An enzyme is a protein which functions as a catalyst of a biologic reaction The enzyme may catalyze a group reaction such as the oxidation of all aldehydes by aldehyde oxidase or it may be absolutely specific to one reaction and one substrate The velocity of a given reaction is dependent upon many factors the two most important of which are the inherent properties of the enzyme itself and the concentrations of reactants

#### *Enzyme Quantity and Activity*

In addition to factors which affect the functional activity of a given quantity of enzyme there are factors which affect the amount of enzyme Since experimentally it is almost always the *activity* rather than the *quantity* of enzyme that is measured information on factors that influence the actual amount of enzyme present is meager

Enzymes are either induced or constitutive Induction may be positive or negative i.e. enzyme formation may be stimulated or repressed by factors such as substrate or product (direct or distant) or by other substances which act as inducers suppressors or activators and may in turn be under specific genetic control

Most enzymes in man and higher animals are constitutive in character as far as is known Their formation is controlled by factors other than the immediate environment their synthesis proceeds without the superimposition of a rate control determined by substrate product or readily altered inducer

Some of the inherited metabolic disorders of man may represent defects in enzyme induction and may therefore potentially be reflections of mutations of modifying or activating genes rather than of the primary gene itself An example may be the Crigler Najjar syndrome of hyperbilirubinemia in which the glucuronyl transferase is underactive This enzyme is normally deficient at birth but increases in activity shortly thereafter Such an increase fails to occur in the syndrome mentioned the failure possibly represents a failure of the inductive mechanism rather than a true absence of enzyme potential

Since enzyme excess is the rule for most reactions it follows that in general a reduction in the amount of enzyme must be considerable before

any limitation of activity becomes apparent indeed the factor of safety may be so great that almost a total lack of the enzyme is required before absence of its function can be detected This explains why it is so often difficult to detect an abnormality in the subject heterozygous for an enzymatic defect It also suggests that these heterozygotes may be detected by loading of their limited amount of enzyme with excessive substrate a device frequently employed

### *Qualitative Change in Enzyme*

The net effect of a qualitative change in an enzyme may be exactly the same as a change in amount of enzyme synthesized because it is only the activity of the enzyme that has relevance to the economy of the organism Severe damage or deletion of a gene may result in complete failure to elaborate the enzyme under its control A lesser genetic change may result in formation of an enzyme protein with abnormal structure Such an alteration might change the function of the enzyme only slightly, if at all but it is apparent from the example of the hemoglobins [28] that a seemingly trivial change such as the substitution of one amino acid in a lengthy chain may have a drastic effect on catalytic function Indeed a *Neurospora* mutant has already been found which contains a protein indistinguishable antigenically from the enzyme tryptophan synthetase but which is enzymatically inactive, presumably the result of just such a minor and yet critical alteration in structure [30]

A number of examples exist in man of missing enzyme activity but thus far an inactive enzyme protein responsible for one of the inherited metabolic diseases has not been isolated and compared with its normal counterpart for structural flaws This is a formidable task geared to the pace of advances in the techniques for demonstrating not only protein structure but the relation of structure to function and for further isolating and identifying proteins presently recognized only by their enzymatic activity These means will be obtained in good time however and it is reasonable to predict that soon an 'inborn error of metabolism' of man or mold will be convincingly demonstrated to derive from a change in the responsible enzyme molecule not more complex than that which distinguishes hemoglobin S from the normal pigment

### CONSEQUENCES OF ALTERED PROTEIN SYNTHESIS

The consequences of a genetic change and an alteration in quality or quantity of a protein will depend upon the role normally subserved by that protein As indicated above one can generalize on this theme far beyond the role of proteins as enzymes to consider also their roles in structure and in transport phenomena Disabilities based solely on structural defects will not be considered in this volume even though numerous genetically determined disorders of this type are known A

number of disturbances of circulating proteins which do not function primarily as enzymes will be considered because of their instructive lessons. Included in this group are the disorders of hemoglobin and of plasma proteins. Also a number of disorders to be considered fall into a category suggestive of defects at the level of membrane transport. Perhaps these conditions implicate malfunctions of carrier substances serving quasis enzymatic roles. However the majority of the disorders presently recognized and partially understood and included in this volume may be considered consequences of derangements of enzymatic function of the more conventional type or of defects of rate control of reaction sequences. A brief inquiry into the nature of such derangements will provide an orientation for the chapters to follow [§ 5-7].

### *Blocked Metabolic Sequence*

Let us consider the model reaction sequence  $A \longrightarrow D$  and the control lying genes as follows



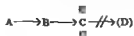
If  $\text{gene}_{CD}$  is defective  $\text{enzyme}_{CD}$  will be inactive and the reaction sequence will be blocked



A number of types of consequences may stem from such a block

1 *Lack of formation of product D or of some more remote product derived exclusively via D* A clinical disorder may result from failure to produce the immediate product D. For example in von Gierke's disease the absence of glucose 6 phosphatase leads to an inability to make glucose from glucose 6 phosphate and hypoglycemia occurs on fasting. Failure of specific steps in thyroxine and hydrocortisone synthesis leads to goitrous cretinism or the adrenogenital syndrome. Alternatively a more remote product may not be formed as in albinism where melanin is lacking because of absence of tyrosinase an enzyme many steps proximate to melanin itself. A secondary consequence of failure to form D may be a regulatory defect if D as an end product is involved in a feed back control mechanism (see below)

2 *Accumulation of C the immediate precursor of the blocked reaction*



This type of consequence may underlie various of the storage disorders such as the glycogen storage disease of liver or muscle due to absence of

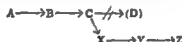
specific phosphorylases. The substrate of the blocked reactions may accumulate in body fluids as bilirubin does when glucuronyl transferase is absent, or in plasma as uric acid does in species lacking uricase. The substrate may accumulate sufficiently to lead to a notable increase in urinary excretion as in the case of homogentisic acid in alcaptonuria or of L-xylulose in pentosuria; prototypes of blocked sequence correctly interpreted by Carro<sup>1</sup>.

### 3. Accumulation of A or B, remote precursors of the blocked reaction



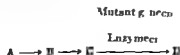
If reactions preceding the block are freely reversible or if the sequence can flow reversibly past unidirectional reactions because of the availability of a second or alternate route, a precursor other than that just proximal to the block may accumulate. In von Gierke's disease absence of glucose 6-phosphatase leads to gross accumulation of hepatic glycogen and elevated blood levels of lactic acid, both several steps removed from glucose 6-phosphate, the substrate of the blocked reaction.

### 4. Production of products normally of minor quantitative importance



A block may result in an increased utilization of normally minor accessory pathways. For example, in phenylketonuria the block in phenylalanine hydroxylation results in an accumulation of phenylalanine and a gross overproduction of phenylketonic products. An analogous situation exists in maple sugar urine disease in which a block in decarboxylation of branched chain amino acids leads to accumulation and abnormal excretion of branched chain amino  $\alpha$ -keto and  $\alpha$ -hydroxyacids. A block in glyoxylic acid metabolism may underlie the enhanced production of oxalic acid in primary hyperoxaluria. In several of the  $\epsilon$  disorders the abnormal product is found excessively in body fluids, but certain disorders characterized by tissue storage lesions may eventually be shown also to be of this general type. The possibility that accentuation of production of ordinarily lesser products involves enzyme induction secondary to accumulation of precursor C may be kept in mind.

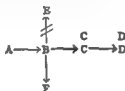
### 5. Production of new product D



If the gene alteration results in production of a functional enzyme differing in character from the normal enzyme perhaps a new product will result. No certain examples of this type of defect exist among recognized hereditary syndromes but perhaps familial primary amyloidosis is a case in point.

### *Defects of Rate Control of a Metabolic Sequence*

In addition to the defects clearly attributable to blocks in known reaction sequences there are a number of disorders which appear to represent alterations of rates of normal reactions or reaction sequences. Perhaps in some cases the apparent defect in rate control is a consequence of a partial or complete block in a side reaction of an early substrate leading secondarily to an increased utilization of that substrate in the reaction pathway to which attention has been drawn.



For example a block in reaction  $\text{B} \longrightarrow \text{F}$  might be recognized primarily as an augmentation of reaction sequence  $\text{B} \longrightarrow \text{C} \longrightarrow \text{D}$ . Over and above this type of situation in which a mistaken interpretation of the nature of the defect might be made there may be disorders representing more valid examples of primary derangements or variations of primitive rate control mechanisms. By primitive is meant the type of control mechanism that might be operative in a single-celled organism or within single cells of a mammalian organism upon which neural or hormonal influences may be superimposed [31]. A few disorders based on defects of hormonal control have been included in this volume when the disorder is clearly hereditary e.g. diabetes mellitus but in the present discussion they will not be further considered.

It is probable that the regulation of metabolism operates always through variations of reaction velocities. A number of types of primitive control mechanisms may be mentioned and at each level one may consider the potential consequences of a quantitative or qualitative change.

1 *Entrance of metabolite into the cell.* Every membrane may be considered a diffusion barrier. Substrates may cross this barrier with variable degrees of hindrance and the entrance of certain metabolites may be under enzymatic control. The enzymatic control may be of several types including that of specific enzymes affecting membrane function such as permeases, enzymes functioning as carriers in active transport phenomena or enzymes effecting removal of the substrate within the cell so as to

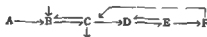


create or sustain a diffusion gradient. A defect at this level has been suspected in Hartnup disease and may account for various of the disorders of renal tubular transport such as cystinuria and renal glycosuria.

2 *Substrate concentration* As pointed out above, in many reaction sequences the enzyme activities are sufficient to permit the sequence to operate at a rate well above its usual functioning level. An example is urea synthesis in man, which can increase considerably with protein loading. In such cases rate control is vested in the concentration of the initial substrate of the chain. It has been suggested that the increased purine synthesis of primary gout may represent a consequence of increased availability of glutamine due to some block in amino acid metabolism [7] or of phosphoribosyl pyrophosphate. The levels of glutamine and PRPP may control the rate of synthesis of the key purine precursor, phosphoribosylamine, and therefore be controlling with respect to uric acid production.

3 *Enzyme activity and the rate-controlling step* When a reaction sequence operates at capacity, its velocity will be determined by the slow step, which then becomes rate limiting. Frequently the rate-limiting step is early in the chain, but it need not be. For example, in conversion of glucose to lactate in intact ascites tumor cells [32] or in leukocytes [33] hexokinase is limiting, and in glycolysis from endogenous glycogen in HeLa cells, phosphorylase is limiting [34]. However, in glycolysis in extracts of HeLa cells [34] the rate-limiting step may be phosphofructokinase or triose phosphate dehydrogenase, depending on growth conditions of the HeLa cells. The rate limitation may reside in either the enzyme quantity or cofactor availability of the key reaction.

4 *Feed back control* In many biosynthetic pathways the rate of the reaction sequence is under control of an end product [35-37]. In all known examples the control operates at the first unidirectional (irreversible) reaction concerned uniquely with synthesis of the product ( $C \rightarrow D$  in sequence  $1 \rightarrow F$ ).



Feed back control is of two types. In the first, the activity of the critical enzyme is regulated by the end product by a competitive type of inhibition: the product and substrate compete for a binding site on the enzyme, creating a chemical control designed to maintain constancy of supply of the product. In the second, the rate of synthesis of the critical enzyme is controlled by the product, which usually acts to inhibit enzyme synthesis but may act to augment it. Thus this type of control may be repressive or inductive. The first type permits rapid adjustments of velocity of a metabolic sequence by environmental factors; the second

a slower regulation. Mutational phenomena may lead to alterations of several theoretical types. Enzyme<sub>CD</sub> might function normally in conversion of  $C \rightarrow D$  but might no longer be inhibited by  $F$  or might be inhibited abnormally by some other product. The biosynthetic capacity for making enzyme<sub>CD</sub> might be essentially normal but the stimulatory or repressive effect of regulatory metabolites might be changed or the biosynthesis of enzyme<sub>CD</sub> might be abnormal because of excessive response positive or negative to normal regulatory metabolites. Possible implications of feed back control mechanisms are suggested in the defects leading to gross overproduction of orotic acid in orotic aciduria and of uric acid in primary gout.

5 *Cytoarchitecture and compartmentation*. It is becoming increasingly clear that cellular function is much more than the aggregate of isolated reactions even allowing for their complex interactions and elaborate interlockings [38]. The structure of the cell may be controlling. For example the characteristics of lactic dehydrogenase are quite different in solution and in contact with microsomes [39]. Also ATP in mitochondria may not be freely available for use by soluble enzymes [34]. Thus structural alterations within cell particles or of intracellular membranes may have important influences upon reaction velocities. In congenital spherocytosis the de Toni Fanconi syndrome, familial periodic paralysis and nephrogenic diabetes insipidus these considerations have been raised they reemphasize the critical role that cytoarchitecture may play in the orderly behavior of metabolic processes. In a group of disorders suggesting end-organ failure such as nephrogenic diabetes insipidus, pseudo hypoparathyroidism and vitamin D-resistant rickets the possibility of a missing or abnormal binding protein necessary for local fixation of the hormone or vitamin must be considered.

### *Pleiotropism and Phenocopy*

There remain a number of situations in which a single genetic lesion gives rise to a diversity of phenotypic expressions. Four mechanistic explanations for pleiotropism may be given.

1 The mutation leads to failure of synthesis of an enzyme or other protein which acts as a catalyst, inducer or suppressor of synthesis of one or more additional enzymes.

2 The genetic defect gives rise to an abnormal material which in turn affects nonspecifically many functions and structures. Sickle-cell hemoglobin is such a material.

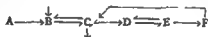
3 The defect gives rise to excessive production of a material which acts with some specificity to inhibit other apparently unrelated biosynthetic sequences. For example phenylalanine and phenylketone products may act as inhibitors of other reactions (cf Chap. 10) thus giving rise to defects of indole and of melanin production.

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geal infections Alcaptonuria gout and even Gaucher's disease, may at times pose very little threat to a reasonably normal existence

**Severe to Lethal** On the other hand many of these conditions have severe consequences to the patient and may even be lethal Branched chain aminoaciduria and untreated phenylketonuria are associated with irreversible changes in the central nervous system The same is true of several of the lipid storage diseases which are often fatal in infancy The retarded development of congenital mutant cretinism may be irreversible Severe hepatic and neurologic disturbances accompany Wilson's disease and many of the organ systems of the body are severely damaged in hemochromatosis The number of inherited metabolic disorders which are manifested only as abortion or stillbirth is of course unknown

### *Age of Onset*

While the fundamental defects in these disorders must be congenitally present, they do not necessarily become clinically evident at an early age The relationship of the altered metabolic function to the phase of growth and development and the operation of other factors balancing the defect may affect greatly the age at which it is recognized Failure to thrive and retardation in development may be apparent almost from the day of birth in patients with branched chain aminoaciduria phenylketonuria galactosemia or metabolic cretinism Niemann Pick disease and Tay Sachs disease are commonly recognized within the first 6 months of life and the adrenogenital syndrome within the first several years

The majority of the hereditary metabolic diseases on the other hand become fully manifest only in the adult Several such as gout hemochromatosis or familial periodic paralysis rarely occur before the third decade Conversely some of these conditions improve as the patients become older Galactosemic patients if they can be helped through their early years with lactose free diets gradually develop the ability to metabolize galactose although their UDP galactose 4-epimerase activity remains permanently missing For unknown reasons patients with adynamia episodica hereditaria and periodic paralysis also may cease having attacks in their later years

### *Effects of Sex*

Some of the inherited metabolic disorders are sex linked such as hemophilia which appears almost exclusively in males and is transmitted by the female carrier Others are sex limited for reasons not always understood For example there is at present no good explanation of why gout occurs so predominantly in males On the other hand hemochromatosis is undoubtedly far more common in males because females have in the menstrual cycle a means of decreasing the iron available for tissue storage

4 The genetic defect may affect synthesis of peptide segments utilized in the eventual assembly of more than one protein. A simultaneous lack of xanthine oxidase and a defect of renal transport of purines have been proposed in xanthinuria [40]. The dual etiology of hyperuricemia in gout [41] raises the possibility of defects of both a biosynthetic enzyme and a renal tubular carrier involved in uric acid transport.

The converse situation—that of independent mutations and different metabolic abnormalities giving rise to disorders of apparent or even real similarity (phenocopy) is not difficult to comprehend. Whether this is achieved experimentally by inducing a metabolic lesion in animals or whether it occurs spontaneously in man in the form of superficially identical disorders, the principles to be considered are the same. In any complex and interlocked metabolic sequence a number of defective loci may give rise to the same product deficiency or substrate accumulation or to abnormalities of the same protein.

## EXPRESSION OF THE INHERITED METABOLIC DISORDERS

There is great variability in the expression of different inherited metabolic defects and even in the manifestation of one single defect in different individuals. There are several points of reference from which such variability can be compared, perhaps the simplest being the degree of incapacitation of the affected individual.

### *Severity*

**Asymptomatic and without Consequence** Many of these disorders can be classified simply as metabolic 'variants' which are discovered accidentally. Examples of such trivial but informative conditions which are difficult to classify as actual diseases are pentosuria, most cases of fructosuria,  $\beta$ -aminoisobutyric aciduria, albumin II anomaly, and the curious inherited differences in ability to taste the thiourea derivatives [42].

**Asymptomatic Except for Accidental Circumstances** Some of the inherited diseases become symptomatic only occasionally and then quite often because some accidental environmental circumstance has intervened. Trauma is required for expression of many of the inherited deficiencies in blood clotting factors. Several other diseases, such as primaquine-induced hemolytic anemia, are revealed only by exposure to drugs, and others such as acute hepatic porphyria may be gravely aggravated by them.

**Mild to Moderate** The inherited diseases are sometimes troublesome and symptomatic but compatible with long life and productivity. Patients with familial bilirubinemia are relatively asymptomatic unless they develop biliary stones. Acatalsia seems benign in the absence of nasopharyn-

## GENETICS AND EUGENICS

*Genetics and Incidence*

Since inherited metabolic diseases cannot be cured in a true sense the reduction of morbidity becomes mainly an exercise in eugenics. Perhaps ultimately the prevention of harmful mutations will become a clearly defined public health discipline but it would be premature to devote space here to the present ragged outlines of this problem. Of practical importance at the moment are means to decrease the clinical manifestations of existing mutations.

Most of the diseases discussed in this volume have an autosomal recessive pattern of inheritance. Care to exclude consanguineous marriages in tainted families will cause these diseases especially those with low gene frequency, such as phenylketonuria to become increasingly rare. A lesser effect will be noted of course on the incidence of diseases with greater gene frequency such as drug induced hemolytic anemia.

Diseases with an autosomal dominant mode of inheritance generally are limited in incidence only by any decline in fecundity of patients with the disease. Here too the frequency of the disease in offspring and possibly a greater degree of severity in the presence of a double dose of the offending dominant gene are factors which may be influenced by the avoidance of doubly tainted matings.

*Detection of Heterozygotes*

The preceding paragraphs indicate the desirability of detection of the heterozygous genotype. Considerable success has already been achieved. Tests depend mainly upon detection of a failure of the enzyme to handle an abnormal load or even a normal load of substrate. Thus in phenylketonuria it has been shown not only that there is reduced tolerance to an administered load of phenylalanine but that there is actually a slight elevation of the phenylalanine level in the blood of heterozygotes. Heterozygotes of many other diseases have now been detected as will become apparent in subsequent chapters. Improvement of detection techniques undoubtedly will continue but in many instances the detection of reduction in enzyme activity in the heterozygote may be quite impossible except by direct analysis.

*Eugenic Counseling*

Already biochemical methods are available for fairly accurate detection of heterozygotes in several conditions. As more tests become refined and available they will become important in marriage counseling [44]. Even if the delicate equilibrium of the courtship  $\rightarrow$  marriage equation fails to be influenced by unfavorable genetic information the lives of offspring

## THERAPEUTIC POSSIBILITIES

These diseases lend themselves unusually well to the ingenious employment of therapeutic maneuvers and often the results are at least as satisfactory as in the commoner diseases. The type of approach varies with the nature of the defect and the means available to overcome it.

*Limiting the Intake of a Precursor Which May Undergo Toxic Accumulation*

It is sometimes possible to withhold from the diet a substance whose metabolism is blocked and which is toxic or which gives rise to toxic metabolites through alternative metabolic pathways. Considerable success has attended the treatment of phenylketonuric children with diets low in phenylalanine. Phenylalanine itself is essential in only minute amounts providing tyrosine is in adequate supply and tyrosine being beyond the block is metabolized normally. Galactosemic children improve remarkably on lactose free diets and the toxic form of fructosuria can be prevented by avoiding fructose.

*Supplying the Missing Metabolites*

When the metabolic block prevents formation of sufficient end product, the disease can sometimes be alleviated by supplying the needed metabolite. The macrocytic anemia of the single case of orotic aciduria so far reported was relieved by feeding cytidylic and uridylic acids. Much can be done to ameliorate metabolic cretinism if thyroxine is provided sufficiently early. Administration of cortisone inhibits the stimulated pituitary of the adrenogenital syndrome, with gratifying relief of signs and symptoms.

*Environmental Manipulation*

The therapeutic resource in the group of patients with metabolic idiosyncrasy is obvious once the diagnosis has been made. The avoidance of drugs known to induce hemolytic anemia in sensitive patients is effective and readily achieved. Likewise the avoidance of cholinesterase inhibitors in sibships where hypersensitivity has been demonstrated [43] and of barbiturates in sibships in which there is a case of porphyria are to be strongly recommended.

*Depletion of Stored Substances*

It is sometimes possible to deplete the body of a storage substance with toxic properties. Thus with British anti-lewisite (BAL) or with penicillamine copper can be removed from the body with benefit to the patient with Wilson's disease. Much can be done for hemochromatosis by frequent phlebotomy and classically uricosuric substances such as probenecid and salicylates are beneficial in gout by increasing the elimination of uric acid.





affected with a condition like galactosemia or phenylketonuria can be greatly altered by early recognition and treatment made possible by such information

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when they recently examined human tissue cultures Chu and Giles [4] found that the 34 subjects which they studied all had 46 chromosomes. Kodani [5-6] has reported the existence of three chromosome numbers—46, 47, and 48—in gonadal tissues and has suggested that the chromosomes in excess of 46 are supernumerary. The controversy over this once closed question has generated much interest. A photograph of metaphase chromosomes from a human somatic cell (with 46 chromosomes) is shown in Fig. 2.1.

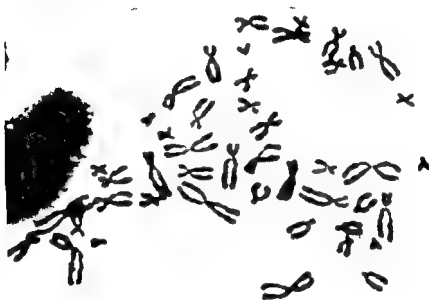


Fig. 2.1 Human metaphase chromosomes prepared from a culture of connective tissue (Courtesy of J. Lejeune)

Two recent findings illustrate the importance which cytologic studies of chromosomal status (karyotype) may have in the recognition and understanding of certain diseases. In some persons with Klinefelter's syndrome an extra chromosome has been found giving a total of 47 [7-8]. This extra chromosome resembles an X chromosome morphologically and these persons are presumed to have two X's and a Y chromosome. The low frequency of X-linked red-green colorblindness (a recessive trait) in Klinefelter's syndrome is consistent with the presence of two X chromosomes [9]. Analysis of the karyotype of mongolian idiots has also revealed the presence of an extra chromosome [10-11]. In this case the supernumerary chromosome would presumably be an autosome.

Two general mechanisms of chromosomal replication and division are known. In mitosis the duplication of somatic cells each chromosome

## Chapter 2

### Human Heredity and Its Biochemical Bases\*

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*II Eldon Sutton*

#### CYTOLOGIC BASIS OF INHERITANCE

The cytologic experiments of the last part of the nineteenth century provided the basis for the proposition that chromosomes are the structures responsible for the transmission of inherited traits. These elongated nuclear bodies are governed in their replication by strict rules which ensure that each daughter cell receives the same complement of chromosomes as the parent cell possessed. Although the chromosomal theory of inheritance has been amplified in many details, its general principles have remained unchanged.

In sexually reproducing higher organisms each somatic nucleus usually possesses a double (*diploid*) set of chromosomes, the corresponding members of which are said to be homologous. The germ cells (*gametes*) possess only a single (*haploid*) set. Cells in certain tissues, such as liver, may occasionally possess higher multiples of the haploid number and are said to be *polyploid*. Homologous chromosomes possess similar sets of genes normally arranged in identical linear fashion along the chromosomes. An exception in many animals is the pair of chromosomes which determines sex. Two unequal chromosomes are recognized, designated X and Y. In the human species females have two X chromosomes and males have an X and a Y chromosome. Chromosomes other than the sex chromosomes are referred to as *autosomes*.

The number of chromosomes varies widely among different species but is relatively constant within a species. In man the number was reported by Painter [1] to be 48 (24 pairs). Although this number was confirmed by many observers, Tjio and his associates [2, 3] found only 46 chromosomes.

This work was supported in part by Research Grant A 830 from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service.

These haploid cells are the germ cells (gametes) i.e. spermatozoa or ova. A diploid zygote results from the fusion of a spermatozoon and an ovum.

Although the distribution of chromosomes into daughter cells is random, the genes which are together on the same chromosome must obviously be transmitted as a group. An exception to this occurs in the case of crossing over (referred to above). The genes which are on the same chromosome are said to be *linked* and form a *linkage group*. The number of linkage groups would correspond to the haploid number of chromosomes in an organism. Since the genes are linearly arranged on the chromosomes, the frequency

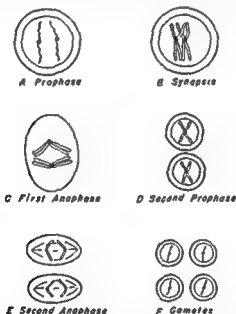


Fig 2-3 Meiosis. A single pair of chromosomes is shown. A Prophase I, B synapsis, C first anaphase, D second prophase, E second anaphase, F gametes.

with which crossing over occurs between any two genes is a measure of their distance apart. These distances are additive and are expressed as the per cent of *recombinants* (exchanged marker genes) found in the offspring of a mating involving two such recognizable genes. Genetic distance does not show a 1:1 correspondence with physical distance although the two are related.

For most organisms which have been studied extensively linkage maps can be constructed showing the position of each observed gene along a one dimensional scale. In the case of man, virtually all the known genes which can be assigned to a linkage group occur on the X chromosome. There are over 40 such genes including those causing red green colorblindness and classical hemophilia. Various mutant genes have also been reported to

reproduces itself and then splits lengthwise the two daughter chromosomes migrating to opposite ends of the cell which subsequently divides. This process is illustrated in Fig 2.2. The important point to note in mitosis is that each daughter cell receives a full complement of chromosomes exactly like those of the parent cell from which it was derived. Microscopic similarity of course is no proof of identity at the molecular level but there is evidence which suggests that the identity does extend to the molecular level.

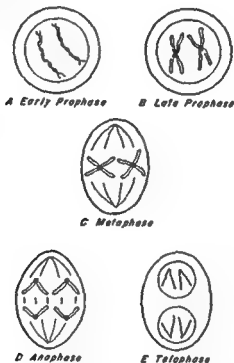


Fig 2.2 Mitosis. Only a single pair of chromosomes is shown. A Early prophase B late prophase C metaphase D anaphase E telophase

**Meiosis:** the formation of germ cells is analogous to mitosis as shown in Fig 2.3 but there are some important differences. Prior to separation of the daughter chromosomes (*chromatids*) the homologous chromosomes pair (*synapse*) lengthwise. During this synapse bridges (*chiasmata*) are sometimes formed frequently resulting in a reciprocal exchange (*crossing over*) of homologous parts of the chromosomes. This is a most important process in that it provides the species with new combinations of genes some of which may have selective advantage. Following synapsis the homologous pairs separate and migrate to opposite ends of the cell which then divides. Without further replication the two cells again divide producing four daughter cells each with a haploid number of chromosomes.

These as well as other criticisms have been answered by very highly purified transforming principles prepared more recently. For example Hotchkiss [21] has obtained an active transforming preparation for pneumococcus which contains less than 0.02 per cent protein. In addition crystalline pancreatic DNase in extremely low concentrations ( $10^{-4}$  mg per ml) rapidly destroys the ability of an extract to transform [22]. It is difficult to avoid the conclusion that the active part of the transforming principle is indeed DNA.

Evidence has also been obtained from studies of bacteriophage infection that DNA carries the genetic information. This work is embodied in the classic paper by Hershey and Chase [23]. It had been established that infective phage particles consisted of two substances—DNA and protein. These workers grew the phage particles on  $P^{32}$  labeled bacteria in order to label the phage nucleic acid and on  $S^{35}$  to label the phage protein. In a series of ingenious experiments they demonstrated that the phage particles consist of a core of DNA surrounded by a protein coat. When the phage particles adsorb onto a sensitive bacterium the DNA is 'injected' into the bacterial cell the protein coat remaining outside. If a strong shearing force is applied to the phage bacterium combination the protein coats (minus their DNA) can be recovered and shown to retain all the antigenic properties associated with the intact phage. Removal of these protein coats from the bacteria after the DNA has entered the cells does not alter the course of the infection of the bacteria by the phage. In these experiments a small amount of protein—about 3 per cent of the injected mass—actually does enter the cell. This very small amount however is not thought to play a major role in the transfer of genetic information.

An apparent exception to the rule of DNA as the genetic material is found in the plant viruses. The extensively studied tobacco mosaic virus for example consists of a long core of RNA surrounded by a cylinder of protein. By careful treatment of the tobacco mosaic virus Fraenkel Conrat et al. [24] have succeeded in separating the RNA from the protein and recombining it with other strains of tobacco virus. The resultant combination is infective but produces progeny with characteristics of the strain from which the RNA was obtained rather than the strain from which the protein was obtained. Gierer and Schramm [25] have treated tobacco mosaic virus to obtain RNA preparations which retain part of the infectivity of the intact virus. In these plant viruses the RNA appears to take over the role occupied by DNA in other organisms.

#### STRUCTURE OF DNA

The building blocks which make up the nucleic acids have been recognized for many years but the manner in which they are put together has been a most difficult puzzle to solve because of the immense size of the intact nucleic acid molecules (molecular weights greater than ten million).

occur on the Y chromosome, but as discussed later the existence of any gene on the Y chromosome has been questioned [12] The only linkages of autosomal genes which are generally accepted are between the Rh factors and elliptocytosis [13, 14] the ABO groups and nail patella syndrome [15] and the Lutheran group and secretor type [16-17] Evidence strongly suggesting linkage between other factors has been published An important fact to note is that two linked genes will show a correlation within single pedigrees but not within the population as a whole Morton has suggested the existence of two genes for elliptocytosis since this trait was linked to the Rh blood groups in only four of the seven large pedigrees which he studied [14-18]

## CHEMICAL STRUCTURE OF GENETIC MATERIAL

### DNA AND GENETIC INFORMATION

Chemical studies of nuclei both by histologic techniques and by assay of isolated nuclei have consistently revealed the presence of three major organic materials These are ribonucleic acid (RNA) deoxyribonucleic acid (DNA) and protein including a large amount of basic protein The chromosomes consist primarily of DNA and protein which are presumably combined as nucleoprotein

It was long assumed that the protein moiety was the carrier of the vast amount of genetic information necessary ultimately to specify the structure of all the proteins synthesized in a living cell As experimental methods were improved it became obvious that the DNA should also be considered as a possibility

The first critical experiments to demonstrate that DNA can in fact transmit genetic information were carried out by Avery, MacLeod and McCarty [19] As early as 1928 Griffith [20] reported that killed pneumococci of one capsular type when injected into mice simultaneously with live pneumococci of another capsular type could lead to the recovery of live pneumococci whose capsular type matched that of the killed cells Avery and his coworkers extended these results by the preparation of extracts which had the transforming potency of the original bacterial cells They concluded that this transforming principle was DNA for the following reasons (1) quantitative elemental analysis showed it to resemble DNA (2) chemical studies revealed the presence of DNA (3) with the physical means available only a single DNA like substance was detectable (4) there were no antigenically active substances present and (5) deoxyribonuclease was the only enzyme among those tested which destroyed the transforming activity To be sure the presence of very small amounts of non DNA material could not be excluded, and the DNase available to these workers was not known to be free of other enzymes hence the possibility still existed that the genetically important substance was not DNA

of more than one polynucleotide chain with some information on the main dimensions of the helix (3) the approximate 1:1 molar ratio of adenine to thymine and guanine to cytosine suggesting that these bases occur in pairs and (4) the probable joining of the bases by hydrogen bonds. Watson and Crick were able to construct a model which conformed to these findings and which agreed very nearly with the x-ray diffraction data obtained from studies of crystalline DNA. Their model shown schematically in Fig 2-4 consists of a right handed double helix formed by two polynucleotide chains which are relationally coiled. The 'backbones' of the chains, the sugar phosphate structure, are on the outside of the helix and the bases are on the inside of the cylinder. A particular merit of the Watson-Crick model is the specific pairing by hydrogen bonds of adenine with thymine and guanine with cytosine.

Watson and Crick recognized that this model had several properties which could be used to explain certain genetic events [29]. First the DNA chain could store information by virtue of the choice of four nucleotides at each nucleotide position. Second each individual strand could direct the replication of its reciprocal structure. If we picture the helix uncoiling with separation of the strands, then each strand could serve as a template upon which free nucleotides could condense in a specific order. These free nucleotides could then polymerize yielding two separated but identical double helices. An interesting point in this model is that the daughter helices have an end from homologous templates which are reciprocal rather than identical.

In only a few instances such as in bacteriophage is it perhaps proper to think of the chromosome as a naked piece of DNA. The usual situation is to have the DNA in combination with proteins, in particular the basic histones. The exact nature of this combination is not known, nor is the arrangement of DNA within the chromosomes of higher organisms. Whether all the DNA in chromosomes is active genetically is yet to be ascertained.

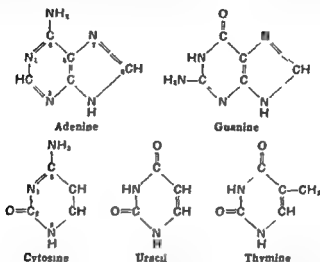
Of great interest has been the recent discovery of an unusual type of DNA in the bacteriophage  $\phi\lambda$  174 [29a]. Physical studies indicate that this DNA consists of a single polynucleotide strand, i.e. one half of a Watson-Crick double helix. The molecular weight of 2 million is considerably lower than that of ordinary DNA. In many respects this single-strand DNA seems to be analogous to RNA which also is a single strand.



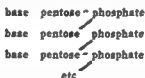
Fig 2-4 Diagram of double helix of DNA. The double bonds indicate hydrogen bonding between specific base pairs. (By permission of J. D. Watson and F. H. C. Crick [7].)



The basic units in nucleic acids are the nucleotides consisting of a purine or pyrimidine base, a pentose (ribose or 2 deoxyribose) and phosphate. Five bases are commonly found in nucleic acids: the purines adenine and guanine, and the pyrimidines cytosine, uracil, and thymine. Occasionally 5-methylcytosine may replace part of the cytosine, and in some bacteriophages 5-hydroxymethylcytosine replaces all the cytosine [26].



In the nucleotides the bases are attached by the N9 (purines) or N3 (pyrimidines) to the C1 hydroxyl of the pentose (furanose structure). The phosphate is attached to the C5 hydroxyl. In the polymerized form an additional bond is formed between the phosphate of one nucleotide and the C3 hydroxyl of the sugar in the next nucleotide, thus:



There are several differences in chemical composition between the two types of nucleic acid. Ribose appears in RNA and 2 deoxyribose in DNA. An equally distinct difference occurs in the bases which comprise the two nucleic acids. Although adenine, guanine, and cytosine are in both types, uracil occurs only in RNA and thymine only in DNA.

In addition to this chemical knowledge of nucleic acid structure, certain other facts had been established prior to the presentation in 1953 by Watson and Crick [27] of their molecular model of DNA. As reviewed by Wilkins [28], these were (1) the dimensions of the nucleotides (bond lengths, etc.) (2) the existence of a helical structure probably consisting

and if these segments do not overlap then by breaking and rejoining the two chromosomes at a point between the deletions one obtains one intact chromosome and one chromosome containing two deletions. However if the deletions on the two chromosomes happen to overlap it is not possible to reconstruct a normal chromosome by this method. This principle is valid also if one (or both) of the mutations is a point mutation rather than a deletion. In the case of point mutations some normal progeny will result from mutations back to wild type and this number must be measured and subtracted from the total number of wild type progeny obtained from a cross of two different mutants. By ascertaining which deletions and point mutations are able to yield normal recombinant offspring and by measuring the frequency of recombinant offspring produced by various

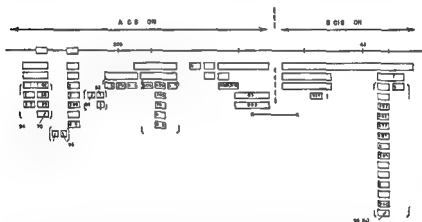


Fig 2 5 Preliminary locations of stable *rII* mutants. Mutants producing no wild recombinants with each other are drawn in overlapping configuration. Pairs which produce small amounts are placed near each other. (By permission of S Benzer [33].)

pairs of mutants it is possible to establish a unique linear arrangement of the mutant loci. In bacteriophage the formation of recombinants probably does not involve breakage and reunion of the parental chromosomes (or DNA molecules). Rather it seems to arise from a switch-over from one parental template to another on the part of the forming offspring molecule. The consequences of this process are geometrically the same as for crossing over in higher organisms. Figure 2 5 is a diagram of some of the deletions found at the *rII* locus of bacteriophage T4.

The accurate mapping of closely linked point mutations requires the examination of many progeny but with bacteriophage this is a feasible undertaking. The first step is to determine in what segment of the *rII* locus the point mutation is located. This is most conveniently done by studying its ability to recombine with the known deletions. After the mutation is located within a segment defined by a particular deletion its frequency

of approximately this molecular weight. This bacteriophage may prove a most useful organism for the study of DNA (and RNA?) function.

### GENETIC MAP STRUCTURE

The ultimate structure of genetic material can be approached by genetic as well as chemical studies, and both approaches have yielded rich rewards during the last few years. The genetic approach is based on the fact that when two chromosomes (or DNA strands) come into close association during replication, occasional progeny will arise in which crossing over has occurred. Crossing over is observed in all higher plants and animals and in most lower organisms. The frequency of recombination of any pair of genes on the same chromosome is proportional to the distance between them, and in fact this distance is commonly expressed as the per cent of recombinants in the progeny of a mating of parents carrying the two gene markers. (One hundred per cent = 100 map units. A linkage group may be greater or less than 100 per cent, although distances greater than 50 per cent must be obtained by addition of smaller distances.) If no recombination occurs, then the two markers are considered to be alleles of the same gene or at least very closely linked. In the latter case, recombination might be so rare as to preclude observing it in organisms with few progeny and long generation times.

Important genetic evidence on the internal structure of genes came with the discovery of "pseudoalleles" [30]. Basically this phenomenon consists in the rare recovery of recombinant wild type progeny which are functionally normal from parents each of which possesses only mutant genes at the genetic locus under study. In other words, two mutant genes are able to recombine and give a wild type offspring even though they are allelic.

The solution to this problem has come only after an extensive series of studies by many workers. The work of Benzer is perhaps the most revealing, because of the power of genetic resolution possible with bacteriophage. The fact that results with other organisms have yielded the same conclusions indicates the generality of these findings [31]. Benzer [32, 33] studied a series of mutants in bacteriophage T4 which grows on *Escherichia coli*. These  $r^+$  mutants are recognized by the larger plaques which they form on sensitive host cells, and they can be separated genetically into three groups depending on the section of the chromosome in which the mutations occur. Benzer has examined the  $rII$  group in detail and has further separated these mutants into two types—those which never show a spontaneous reversion to wild type and those in which an occasional mutation to wild type occurs. The former are best explained as *deletions* of genetic material, while the latter can be considered as alterations at a single point (*point mutations*).

If by chance two chromosomes have each suffered deletion of a segment

functional groups then the nonmutant segments of each mutant complement each other in their activities and both mutants can grow. If on the other hand both mutations are in the same functional group then there is no intact segment capable of carrying out this particular function and neither mutant will grow. Such genetically identified functional units have been given the name *cistron* by Benzer because of their detection by the *cis trans* test of Lewis. It is generally assumed that each *cistron* is responsible for the synthesis of a separate protein (or a polypeptide chain). In the case of the *rII* locus presumably two products are necessary to carry out whatever this function may be. In applying the word *cistron* however it should be remembered that this term is defined with respect to a genetic test and not with respect to any chemical product.

To take care of the two remaining definitions of a gene Benzer has coined the terms *muton* for the smallest mutable unit and *recon* for the smallest unit of recombination. The present methods of genetic analysis permit one to set only an upper limit on the size of these units; nevertheless this limit in both cases has approached the size of a single nucleotide pair.

### REPLICATION OF DNA

A picture of the replication of the DNA double helix has already been presented in which the intertwined chains unwind each then serving as a template on which nucleotides condense and polymerize giving rise to a complementary strand. There are still many problems to be solved in translating this attractive theory into fact among which is the unwinding problem. This problem has not been made easier by the recent report that the true molecular weight of DNA may be of the order of 100 million rather than 10 million as previously thought [34a]. In the case of a bacteriophage particle the DNA is much longer than the diameter of the cell in which it is replicating. Intuitively one would reject the idea of such a tangled mass regularly unwinding and replicating. However calculations by Levinthal and Crane [35] suggest that the energy required is not too great and that perhaps one's intuition is unreliable.

The necessity of unwinding such a large structure is based on the assumption that DNA *in vivo* occurs in the same large pieces in which it is usually isolated and furthermore that these large pieces are not temporarily broken down during DNA replication. Direct proof of this in most organisms is difficult but it has been possible to demonstrate that parental DNA does not become randomized during replication. The same experiments demonstrate that parental DNA is divided equally between daughter cells. Thus there is a mechanism of replication which involves a regular division of the DNA molecules into two equal parts.

of recombination with other mutants within the same segment is ascertained. The results of the analysis of such a series of mutants by Benzer is shown in Fig. 2.6. This series of mutants occurs within a deletion designated *r164*. Of 145 reverting mutants found within this region, 123 occurred at the same point (called *r131* after the first mutant found at this point) the remainder being distributed at 10 different points. This non-random distribution points out the very interesting fact that mutations are likely to occur at certain sensitive points rather than at random.

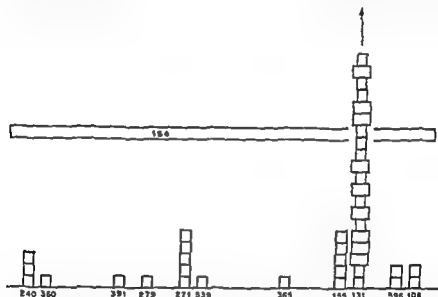


Fig. 6. Grouping into species of mutants within the segment defined by the deletion *r164*. The arrow indicates 103 additional mutants of the *r131* species. The different-size boxes of the *r131* species indicate two distinct varieties based on the reversion rates. The number assigned to each location is that of the first mutant found at that position. (By permission of S. Benzer [33].)

Such a mutation profile may vary considerably with the mutagenic agent, some points being sensitive to one mutagen but not to another [34].

An analysis of genetic fine structure such as just described raises questions concerning the definition of a gene. Traditionally a gene has been defined in three ways—as a unit of function, as a unit of recombination, and as a unit of mutation. In dealing with widely separated markers there is no experimental incompatibility among these definitions. In the *rII* locus of bacteriophage T4, however, there is an illustration of the contradictions which can exist. For example, in mapping the *rII* mutants Benzer discovered that they could be divided into two functionally independent groups designated *A* and *B* in Fig. 2.5. This functional independence can be ascertained by infecting an appropriate strain of *E. coli* simultaneously with two *rII* mutants. If the two mutations are in different

labeled DNA. In accord with the other experiments cited they found that a single replication reduced the label by one half forming hybrid molecules of half  $N^{15}$  and half  $N^{14}$ . Heat denaturation of the hybrid DNA reduced the molecular weight by one half and at the same time permitted recovery of pure  $N^{15}$  and pure  $N^{14}$  subunits. The most reasonable explanation is that denaturation caused the double helix to unwind, yielding a strand of  $N^{15}$  parental DNA and a strand of  $N^{14}$  daughter DNA. Thus the original postulate of Watson and Crick [29] on DNA replication is substantiated.

## GENE ACTION AND SPECIFIC PROTEINS

### RELATIONSHIPS OF NUCLEIC ACIDS TO PROTEIN SYNTHESIS

All known biochemical reactions in organisms depend ultimately on proteins for their catalysis. Of first importance in the study of gene action is the manner in which proteins are synthesized with particular reference to the nature of protein specificity.

The first suggestions that nucleic acids control the synthesis of proteins were made by Caspersson [38] and by Brachet [39] reasoning primarily from their cytologic studies. By the use of such techniques as ultraviolet microspectrophotometry, Feulgen staining and enzyme digestions it was demonstrated that the DNA occurs exclusively in the nucleus while RNA occurs both in the nucleus and in the cytoplasm. Furthermore the localization of DNA in the chromosomes and its regular increase and distribution with cell division suggested DNA as an important part of the primary genetic system [40]. RNA on the other hand varies among different cell types within an organism and is the nucleic acid present in the nucleolus. If one compares the amount of RNA in different cells with the rate of protein synthesis by those cells a good correlation is obtained. The silk gland of the silk worm has the highest RNA content known. Liver and pancreas have a very high RNA content while heart muscle and kidney cells have a low RNA content. The rates of protein synthesis in these last are low although their metabolic activity is high. This association suggests a relationship between RNA level and protein synthesis [41].

Since the work of Caspersson and of Brachet many experiments have been performed with the purpose of demonstrating the association of nucleic acids and protein synthesis. Most of the earlier conclusions were based entirely on circumstantial evidence. With the preparation in recent years of cell free systems able to incorporate amino acids into proteins the study of protein synthesis has been put on a firm experimental basis which is yielding results truly valid at the molecular level. These will be reviewed briefly although it is apparent that there are still tremendous gaps in our knowledge.

One of the first experiments to show that DNA replicates as a unit was conducted by Levinthal [36] who grew 12 bacteriophage on *E. coli* which were heavily labeled with  $P^{32}$ . The intensity of labeling of DNA in the phage was sufficient for individual molecules to be studied with the autoradiographic technique. If the original labeled phage were ruptured by osmotic shock so that the DNA was released into solution, approximately 40 per cent of the total label was found to remain intact in large pieces of DNA (molecular weight about 4.5 million) while the remaining 60 per cent was dispersed as small molecules. If the phage were allowed to replicate in unlabeled bacteria for one or two generations the labeling was found still to be associated with a few intensely labeled particles. However, each labeled particle now contained only 20 per cent of the original label.

A recent study by Taylor et al [37] using labeled thymidine with *Vicia faba* (broad bean) is of interest because it shows the same results in this higher plant. Thymidine is incorporated only into DNA and generally only during DNA synthesis. Taylor exposed dividing root cells



Fig 2-7 Replication of a single chromosome showing conservation of the original DNA within a half-chromosome strand. Reciprocal exchange of homologous chromatid segments has occurred at the far right.

to the labeled thymidine for 8 hr (one third of a division cycle) after which they were transferred to colchicine for varying periods of time. Colchicine inhibits cell division without inhibiting chromosome replication. Autoradiographic studies indicated that those cells in which the chromosomes had replicated during exposure to thymidine possessed both sets of daughter chromosomes equally labeled. After an additional replication only one of each pair of daughter chromosomes was labeled. If the cells were allowed to replicate still another time half of the still attached daughter chromosome pairs were unlabeled while half showed a single daughter chromosome labeled.

These two studies provide conclusive evidence that whatever the precise mechanism of DNA replication it is conservative in the sense that half of each DNA complement is transmitted intact generation after generation and at no time during the life of a cell do the DNA constituents become randomly dispersed in the nucleus. This is illustrated in Fig 2-7. These studies do not however tell whether the 'indivisible' unit is a single polynucleotide strand, a double helix or some more complex structure.

Meselson and Stahl [37a] have obtained evidence that the indivisible unit of *E. coli* DNA is most probably a single polynucleotide strand. In experiments somewhat analogous to Taylor's they used  $N^{15}$  to label DNA and ultracentrifugal techniques to differentiate labeled from un-

labeled thymidine for 8 hr (one third of a division cycle) after which they were transferred to colchicine for varying periods of time. Colchicine inhibits cell division without inhibiting chromosome replication. Autoradiographic studies indicated that those cells in which the chromosomes had replicated during exposure to thymidine possessed both sets of daughter chromosomes equally labeled. After an additional replication only one of each pair of daughter chromosomes was labeled. If the cells were allowed to replicate still another time half of the still attached daughter chromosome pairs were unlabeled while half showed a single daughter chromosome labeled.

appears in the nondialyzable deoxycholate soluble portion of the microsomes. Since the RNA of the microsomes is almost exclusively in the small particles, the soluble material would appear to be completed protein.

The above studies on the mechanism of amino acid incorporation have been done primarily with liver microsome preparations. It has recently been demonstrated that labeled amino acids can be incorporated also into mitochondria, and that in muscle tissue this may be the predominant site of incorporation [58]. Although mitochondria have not been studied extensively, it may be assumed that similar mechanisms of protein synthesis occur in both types of organelles, since two equally efficient mechanisms are not likely to have arisen and persisted in the evolutionary contest for survival.

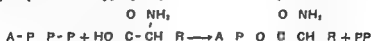
As will be discussed later, the inherited specificity of a protein is a function of the amino acid sequences of the constituent polypeptide chains, and each sequence is rigorously specified. The mechanism for arranging the amino acids into the proper order is still to be studied experimentally, but Spiegelman has presented an impressive argument in favor of a template mechanism rather than a stepwise enzymatic mechanism [69]. In order for a polyezyme system to build up a polypeptide chain one amino acid at a time, it would be necessary that each enzyme have built into it the information of the arrangement of most of the amino acids already incorporated, so that it would act only at the proper moment. This means essentially that each peptide bond of each protein would require a different enzyme. The number of enzymes required would indeed be staggering, particularly if one also considers the number of enzymes required to make the protein synthesizing enzymes.

The alternate template mechanism assumes that the amino acids condense onto specific sites of some large structure where they are then joined together by peptide bond formation. The formation of the peptide bonds could be catalyzed enzymatically, but the enzymes would not be highly specific and would not carry the genetic information. Thus each specific protein, or perhaps polypeptide chain, would originate from a specific template, which would be the intermediate between the DNA of the gene and its product, the protein.

An interpretation of the template mechanism in terms of the present chemical knowledge of protein synthesis is crude; nevertheless, certain analogies can be noted which are most encouraging. From the previous paragraphs it is obvious that the leading candidate for the role of template is RNA. Hoagland et al. [60] have presented a model in which the microsomal RNA acts as the template for the condensation of the soluble RNA-amino acid complexes. The specific arrangement of the complexes is determined by the complementary relationships of the soluble RNA and the large RNA template. Once in position, the RNA moieties of the soluble complexes polymerize to form a large RNA molecule with



The first step in the condensation of amino acids into proteins involves the activation of the amino acids. The formation of a peptide bond requires an external source of energy. Lipmann [42] suggested in 1941 that the reaction proceeds through the formation of amino acyl phosphates. In 1955 Hogland [43-44] discovered soluble activating enzymes which catalyze the reaction between an amino acid and adenosine triphosphate to yield the mixed anhydride of the amino acid and adenosine monophosphate with pyrophosphate as the other product.



The amino acid adenylates are firmly attached to the enzyme and probably do not exist in the free state in biologic systems although they can be separated from the enzymes under special conditions [45-46]. These enzymes are highly specific for a given amino acid and a different enzyme is apparently necessary for each amino acid. Although all tissues have not yielded a complete array of activating enzymes it has been suggested that this is a result of experimental difficulties. The enzymes necessary for the activation of the 20 common amino acids have all been detected in extract from pigeon pancreas [47].

The activated amino acids are next bound to soluble RNA [46-48]. The enzymes which catalyze the transfer of the activated amino acids to RNA are apparently the same as the ones which catalyze the formation of amino acyl adenylates. The process is visualized as the transfer of the amino acyl moiety of the amino acyl adenylate from the activating enzyme directly to soluble RNA [49]. The acceptor sites on the RNA are each specific for an amino acid as indicated by the observation that saturation of the RNA with one amino acid does not affect the ability of the RNA to accept other amino acids [50]. The point of attachment of the amino acids to the RNA is the adenosine group which occurs in the terminal position of soluble RNA [51]. The amino acid forms a covalent ester bond with the 3' (or 2') hydroxyl of the ribose [52]. The terminal adenylate appears to be linked through its phosphoric acid group to the 3' hydroxyl of cytidine [53].

The next steps in protein synthesis are more obscure, and only a few generalizations can be made. It was established several years ago that incorporation of labeled amino acids into liver proteins occurs most rapidly in the microsomes [54-55]. Fragmentation of the microsomes by deoxycholate has revealed the presence of small nucleoprotein particles which appear to be the actual centers for incorporation of amino acids [56]. The reactions leading to the incorporation of soluble RNA-amino acid complexes into the particulate RNA are not known but appear to require guanosine triphosphate [57]. The activity of labeled amino acids reaches maximum values in these particles within a few minutes and gradually

affected in their mutants. This may often be the case but many mutations are now recognized which result in a detectable altered protein product. Perhaps the best known of these in lower organisms although not entirely understood on the chemical level is the series of mutants for tryptophan synthetase in *Neurospora crassa*. This enzyme is responsible for catalyzing the condensation of indole with serine to form tryptophan and mutants lacking the enzyme require the addition of tryptophan to the culture medium. Suckind, Yanofsky and Bonner [66] prepared antibodies to tryptophan synthetase and were able to demonstrate in most of the mutants the presence of cross reacting material (CRM) which lacked enzyme activity. One of the mutants did fail to produce CRM proving the specific association of the CRM with the tryptophan synthetase locus.

Levinthal and Garen (personal communication) have examined a large number of *E. coli* mutants which fail to produce the normal amount of alkaline phosphatase activity under induction conditions. These mutants could be divided into two groups—those in which no enzyme activity could be detected and those in which diminished enzyme activity was present. The relative ease of isolation of the enzyme from this organism made direct examination of the protein possible. These studies still in progress have shown that most of the mutants with diminished activity produce an essentially normal amount of a protein product chemically similar to the wild type alkaline phosphatase but with a lowered catalytic activity. By way of contrast most of the mutants which showed no enzyme activity also produced no protein analogous to the alkaline phosphatase.

A fundamental problem concerns the genetic control of protein differences. At least a partial answer has been found in studies of the hemoglobin mutations in man. The functional properties of proteins are generally considered to result from the nature of the surface such as contour, distribution of charges, etc. This in turn results at least in part from the manner in which the long polypeptide chains are folded. One can imagine that the folding of a polypeptide chain is controlled either by some other surface acting as a template or by the kind and sequence of amino acids which constitute the polypeptide chain. In the case of immune antibodies it has generally been assumed that the specific surfaces result from differences in folding of a non-specific protein blank. Lederberg [66a] has suggested that the evidence is more compatible with the hypothesis that the specificity is due entirely to the primary structure (amino acid sequences) of the antibody molecules.

In the case of the abnormal hemoglobins the specific differences are known to be inherited and have been shown to result from changes in the amino acid sequence. A difficulty in demonstrating this arose from the failure to find reproducible differences in the total amino acid com-

configurational changes which promote the formation of peptide bonds between adjacent amino acids. This last step would destroy the covalent bond between the amino acids and the RNA and the completed polypeptide chain would be free to separate from the RNA. This model is consistent with most of the observations on protein synthesis, particularly the close association with RNA synthesis.

The acceptance of a template mechanism still leaves the question of how the genetic information of the DNA is transformed into the specific structure of RNA. At the molecular level, various models have been proposed but these all assume structures of RNA which are considerably extrapolated from actual knowledge. It is probable that some template mechanism is again involved but the details must await further experimental results.

The site of the information transfer is undoubtedly the nucleus since DNA is not found in the cytoplasm. This is further indicated by such experiments as those of Goldstein and Plaut [61] who inserted  $P^{32}$  labeled nuclei of amebas into unlabeled cells. After several hours there was a transfer of labeled RNA from the labeled nucleus into the unlabeled cytoplasm. With unlabeled nuclei in the presence of labeled cytoplasmic RNA there did not seem to be any transfer into the nucleus. These and other experiments have produced a picture of nucleic acid-protein relationships in which the genetic DNA directs the synthesis of template RNA, the latter diffusing from the nucleus into the cytoplasm where it in turn directs the synthesis of protein. Once in the cytoplasm the RNA at least in some cases can replicate independently of the nucleus [62]. A most interesting demonstration of the independence of protein synthesis from direct participation by DNA is found in enucleated reticulocytes where synthesis of hemoglobin continues after the nucleus has been extruded from the cell [63].

The preceding discussion has dealt primarily with cytoplasmic synthesis of protein. Some protein synthesis also occurs in the nucleus (for example see Allfrey et al. [64]) and as yet the direct intervention of DNA cannot be ruled out. In particular it would seem possible that synthesis of the basic proteins which are part of the chromosomal structure might be under the direct control of DNA. The nuclear enzymes on the other hand are probably controlled by the large amount of RNA in the nucleus.

#### NATURE OF THE PROTEIN PRODUCTS

All inherited differences are expressed eventually in the function of some protein either through changes in the efficiency or specificity of that protein or in the quantity of the protein. Markert and Owen [65] studying a series of tyrosinase mutants in *Glomerella* were unable to detect any enzymatically inactive material which cross reacted with tyrosinase antibodies suggesting that the quantity of enzyme was

for hemoglobin S

val his leu thr pro val glu lys

It is now apparent that the differences in the properties of these two hemoglobins including a difference in solubility which gives morbid consequences in the case of individuals homozygous for hemoglobin S can be traced to a substitution of a single valine residue for a glutamic acid in each half molecule of hemoglobin

In a similar manner hemoglobin C was analyzed and found to be altered only in this same peptide the sequence being

val his leu thr pro lys glu lys

In this case lysine has been substituted for glutamic acid at the same position. It should be noted that glutamic acid is capable of assuming a negative charge, valine is neutral, and lysine can assume a positive charge. This corresponds to the differences in electrophoretic behavior observed with these hemoglobins.

An interesting situation has developed with respect to another abnormal hemoglobin designated D [70]. Samples from three unrelated individuals have been subjected to trypsin digestion; each was found to differ from hemoglobin A in a single peptide, but in each case it was a different peptide. This raises the prospect that in some cases the hemoglobin designations are generic rather than specific. This might be expected since not all changes involving a single amino acid residue in the midst of a large protein molecule would result in readily detectable variations in the behavior of the intact molecule. This is particularly apparent if one considers the limited number of molecular properties available for study. It raises the interesting thought that much undetected inherited variation may exist in normally functioning proteins, and that many genetic mutations may occur which will be detectable only by the types of structural studies which have been applied to the hemoglobins.

#### GENE STRUCTURE VERSUS PROTEIN STRUCTURE

With the demonstration that a mutation can result in replacement of a single amino acid in a polypeptide chain, it is tempting to relate the linear sequence of amino acids in proteins to the linear genetic fine structure of a chromosome. In other words, if one were to map genetically a series of point mutations such as Benzer has done in bacteriophage, and compare the results with the relative positions of altered amino acids in the polypeptide chain which is the product of this genetic locus, would the two series match? There is no answer as yet to this question. One would expect, however, that the nucleotides necessary to specify a particular amino acid would be arranged in the same sequence along the DNA helix as are the amino acids in the polypeptide chain.

position of the hemoglobins. Fetal hemoglobin does have a clearly different amino acid composition from adult but it is produced by a different synthetic and presumably genetic mechanism [67]. In an elegant study of the detailed chemical structure of hemoglobins, Ingram [68, 69] has been able to demonstrate that there is a difference in amino acid composition between normal adult hemoglobin (Hb A), sickle-cell hemoglobin (Hb S),

and the abnormal hemoglobins C and D (Chap. 31). Crystallographic studies had previously shown that hemoglobin probably consists of two identical half molecules. With a molecular weight of 66,700, there would be approximately three hundred amino acid residues in each half molecule. Some thirty of these are the basic amino acids, lysine and arginine. Ingram digested a pure preparation of the particular hemoglobin with trypsin, which hydrolyzes peptide bonds formed from the carboxyl group of lysine or arginine. The resulting mixture of small peptides consisted on the average of 10 amino acids per peptide. This mixture was subjected to electrophoresis on paper and then to chromatography at right angles to the electrophoresis. The results for hemoglobins A and S are shown in Fig. 28. It can be seen that the only difference between these hemoglobins involves a single peptide. All other peptides were indistinguishable and presumably identical.

This interesting peptide was isolated from the two hemoglobins and hydrolyzed to yield the individual amino acids. In both cases, these were found to be histidine, valine, leucine, threonine, proline, glutamic acid, and lysine.

There was a difference, however, in that hemoglobin A contained more glutamic acid than hemoglobin S and conversely hemoglobin S contained more valine. By partial acid hydrolysis a mixture of identifiable smaller peptides was obtained from which a unique sequence could be constructed. For hemoglobin A this sequence proved to be

val his leu thr pro glu glu lys

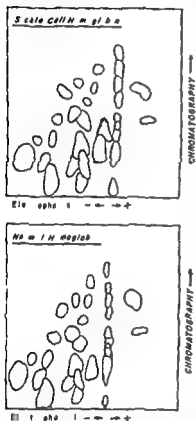


Fig. 28. Polypeptide fingerprints of normal and sickle cell hemoglobins. The shaded spots are those belonging to the peptide in which the amino acid difference exists. (By permission of V. M. Ingram [68].)

ozygote. It is important to note that in this case a single locus controls the specificity of a whole series of proteins and furthermore that the heterozygous individuals produce a series of proteins (hybrid substances) differing from either homozygote. The explanation of this unusual system has yet to come. The interaction of the alleles in the heterozygote—difficult to visualize at the gene level—suggests that a series of polymers may be involved. Thus each allele may control the production of a single monomer, with subsequent reactions independent of this locus producing varying degrees of polymerization. Consistent with this hypothesis is the recent observation of Smithies [81a] that treatment of haptoglobins with sulfhydryl reagents and urea produces protein subunits which correspond more closely to the genes present and may represent the monomeric forms. This hypothesis does not explain all properties of the system.

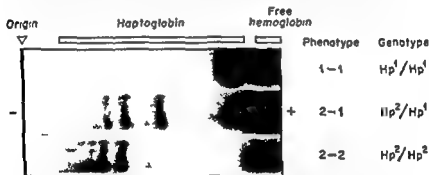


Fig. 29 Plasma electrophoretic patterns obtained with haptoglobins using the starch gel technique. Excess hemoglobin has been added to the plasma to complex the haptoglobins. Benzidine has been used to show the presence of hemoglobin, both bound and unbound. The electrophoresis was carried out in 0.03 M borate buffer pH 8.5, 6 volts per cm for 5 hr.

For example, it does not explain the absence of products of the type 2-2 homozygote in the heterozygous individual. It does, however, suggest an approach which may relate the haptoglobin system to the one gene—one enzyme theory.

In spite of the exceptions which have been taken to the one gene—one enzyme theory, the essential correctness of the idea cannot be effectively challenged [82]. One possibly might modernize it a bit by rephrasing it as one cistron—one polypeptide.

#### GENETIC INFLUENCES ON PROTEIN PROPERTIES

From the preceding sections it is obvious that virtually any property of a protein can be changed as a result of mutation. The quantity, of course, is very frequently changed. It will possibly be of some value at this point to consider some of the qualitative changes which have been demonstrated.

In order for four alternative nucleotides to specify 20 amino acids a minimum of three nucleotide positions must be used to provide the 20 combinations [71]. Three positions with four alternatives each give 64 possible combinations of which many may not specify an amino acid. If a point mutation is pictured as a substitution of one nucleotide for another, then the new combination will sometimes be a "sense" combination and sometimes a "nonsense" combination [72]. Since the latter will be unable to specify any amino acid at its appropriate point in the polypeptide chain then the polypeptide would be incomplete and might not be able to form at all. This type of mutation may account for many of the mutants in which no product analogous to the unaltered protein can be detected.

Implicit in much of what has been noted above is the assumption that each genetic locus is responsible for the synthesis of a specific protein. This idea was effectively expressed by Beadle as the "one gene-one enzyme" theory [73]. This theory has been criticized for many reasons, particularly in view of the developing concept that the dividing line between genes may be much less distinct than originally visualized in the "beads-on-a-string" picture. Recent developments in understanding of hemoglobin structure and genetics are also relevant to this question. First is the demonstration that each half molecule of hemoglobin consists of two different polypeptide chains [74-75]. Second is the report that abnormal hemoglobins S and G are not alleles as evidenced by the fact that a person heterozygous for both S and G produced a child which had only normal hemoglobin [76]. This could have happened only if this G/S individual had a normal alternative for each of these abnormal genes. These two facts taken together have led to the suggestion that the amino acid alterations in hemoglobin G and in hemoglobins S and C occur in different polypeptide chains and that each chain is controlled by a separate genetic locus [77]. This would mean that at least two loci are involved in the production of a single complete molecule. Consistent with the above argument is the observation that hemoglobins S and C involving an alteration at the same amino acid position are allelic [78].

Another system which superficially appears to contradict the one gene-one enzyme theory is the haptoglobins. These are a group of glycoproteins found in human plasma and first detected by Polonovski and Jayle [79]. Haptoglobins are unique because of their ability to combine irreversibly and stoichiometrically with hemoglobin. Smithies [80] with the aid of his high resolution starch gel electrophoresis was able to distinguish three types of individuals each characterized by qualitative differences in his haptoglobins. These three types are shown in Fig. 2.9. A genetic analysis of family data by Smithies and Walker [81] indicated that this variation is controlled by a single locus with two alleles: two of the types representing the two homozygotes and the third the heter

ozygote. It is important to note that in this case a single locus controls the specificity of a whole series of proteins and furthermore that the heterozygous individuals produce a series of proteins (hybrid substances) differing from either homozygote. The explanation of this unusual system has yet to come. The interaction of the alleles in the heterozygote—difficult to visualize at the gene level—suggests that a series of polymers may be involved. Thus each allele may control the production of a single monomer with subsequent reactions independent of this locus producing varying degrees of polymerization. Consistent with this hypothesis is the recent observation of Smithies [81a] that treatment of haptoglobins with sulfhydryl reagents and urea produces protein subunits which correspond more closely to the genes present and may represent the monomeric forms. This hypothesis does not explain all properties of the system.

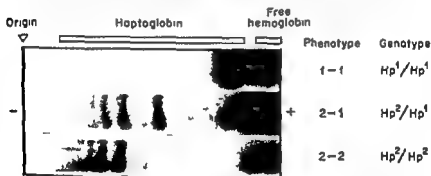


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### Solubility

This property of the protein rarely changes enough with mutation to give rise to observable differences. The one striking exception occurs in man in the case of sickle cell hemoglobin. Although the oxygenated form of hemoglobin S is quite soluble, the nonoxygenated form is sparingly soluble, being only one fiftieth that of hemoglobin A [83]. In well oxygenated blood the hemoglobin S remains in solution, but if oxygen tension is low, the hemoglobin S will form 'tactoids' inside the erythrocyte.

### Temperature Sensitivity

The rate at which a protein undergoes "spontaneous" denaturation is a function of temperature. A number of cases are known in which temperatures compatible with life can cause relatively rapid denaturation of a particularly sensitive protein. One of the more carefully studied

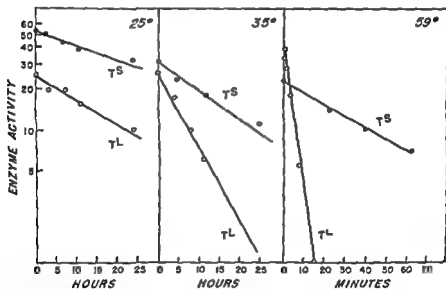


Fig. 2-10 Inactivation of thermolabile (TL) and thermostable (TS) tyrosinase (*Neurospora crassa*) at different temperatures (By permission of A. H. Hovvitt and M. Hing [84]).

occurs in *N. crassa* tyrosinase in a known mutant of this species is very sensitive to temperature. In their experiments with this mutant Horowitz and Hing obtained the inactivation graphs shown in Fig. 2-10 [84]. Other properties of the enzyme such as pH optimum, substrate specificity and sensitivity to cysteine were not altered.

Another example of a temperature sensitive mutant is the Himalaya rabbit, a domestic strain characterized by dark ears, tail, nose, and paws but white body fur [85]. If a portion of the skin is kept below 34°C the

body fur in that area will also be dark. On the other hand if the animals are raised at temperatures greater than 34 C the entire animal will be white. Again the explanation is the presence of a temperature sensitive pigment forming enzyme: presumably tyrosinase. This sensitivity is controlled by a single gene for which other alleles are known and which is recessive to the fully pigmented "wild type" animal possessing stable tyrosinase.

A possible example of temperature sensitivity has been reported in man. As discussed in Chap. 32 approximately 10 per cent of male Negroes will undergo a hemolytic episode when exposed to primaquine and related compounds. This trait is the result of a single gene located on the X chromosome [86-87] and has been traced to a deficiency of glucose 6-phosphate dehydrogenase in the erythrocytes [88]. Studies of this enzyme in affected individuals have suggested that under certain conditions it is more readily denatured by heat than is the enzyme from normal individuals [89]. Such a mechanism would explain the self-limiting nature of the hemolysis. For example it has been established that only the older erythrocytes are sensitive to primaquine [90]. Presumably the younger cells have a full complement of active enzyme which slowly becomes denatured leaving the older cells without defense against the primaquine challenge.

### *Electrophoretic Mobility*

It is obvious that the introduction into proteins of different amino acids will frequently result in alteration of the total charge of the protein molecule and hence in mobility in an electric field. The previous discussion of hemoglobin structure provides a good example of this. Electrophoretic techniques have revealed two axes of genetic heterogeneity in man which were not previously suspected. The first of these was haptoglobin, the details of which have already been presented. A more recent example is that of transferrin: eight different types of transferrin have been demonstrated by means of the starch gel technique [91-94]. Each of these types is the product of a single allele. Homozygous individuals produce only a single type of transferrin whereas heterozygous individuals produce a mixture of two.

### *Immunologic Properties*

It is not possible to discuss in detail the many examples of inherited antigenic differences recognized in man. Most of these occur on the surfaces of cells and have been largely refractory to chemical study, but some progress has been made with the ABO antigens which do occur in a soluble form. These largely carbohydrate substances have not yielded readily to studies of gene action (cf. Kabat [95] for a review of this subject).

A recently described example of an inherited variation in a protein is the  $\eta$  factor of Grubb and Jurell [96, 97] which is recognized entirely by its interference with the ability of certain rheumatoid arthritic serums to promote the agglutination of Rh positive erythrocytes coated with incomplete Rh antibodies. This factor is found in the  $\gamma$  globulin fraction of serums and is either present or absent depending on the genotype of the individual. An immunologic difference has also been reported between hemoglobins A and S, although the magnitude was small [98].

### *Sedimentation Rate*

An exchange of one amino acid for another in a large protein will not affect the molecular weight enough to alter the sedimentation rate in an ultracentrifuge but it may affect the configuration enough to influence the sedimentation and it may affect the formation of aggregates or polymers. Studies on the sedimentation of haptoglobins have revealed a small difference among the three types [99]. One would presume that the differences reflect different degrees of polymerization although whether this in turn is due to the protein or to the carbohydrate moiety is unknown. If it is due to the latter then it is probably not correct to consider this as an alteration in the primary gene product.

### *Other Properties*

It is reasonable to assume that most measurable properties of proteins can be altered by mutation even though examples may not be known at present. Among the properties which should be considered are substrate specificity, Michaelis constant, pH optimum, etc. A distinct difference in any of these properties may be considered good evidence for a mutation.

## GENOTYPE AND PHENOTYPE: EXPRESSION OF INHERITED TRAITS

### REGULATION OF PROTEIN SYNTHESIS

Whatever an individual's genetic make up may be it is expressed only after interaction with the environment. It is often necessary to untangle complex environmental factors before one can bring order to the genetic elements. In addition, the gene products interact with each other, sometimes forming interlocking self-regulating systems whose analysis may be extremely baffling. This section contains a discussion of a few of the systems which have been studied; it is hoped that they will serve as useful models for other systems.

If an organism carries the genetic information necessary to synthesize a specific protein, what then determines how much of that protein is

produced? The existence of regulating mechanisms is everywhere apparent but knowledge of them remains largely at the descriptive level. One of the more interesting control phenomena is that of enzyme induction, sometimes still referred to as adaptive enzyme formation [100]. The ability of yeast to utilize galactose as a source of energy depends on the presence of an enzyme, galactozymase. Yeast cells grown on a glucose medium do not normally produce galactozymase, but if galactose is supplied the galactozymase soon appears. It is important to emphasize that this increase is not due to the selection of genetically different cells which already are producing the enzyme, rather it represents the new synthesis of galactozymase in individual cells which previously did not possess the enzyme. In this sense the culture has become 'adapted' to the medium although no selection has occurred.

This phenomenon is more properly described as 'induction' than 'adaptation'. This is illustrated by the experiments of Monod's group [101]. In order for *E. coli* to utilize lactose it must produce the enzyme  $\beta$ -galactosidase. This enzyme is not ordinarily present in the absence of lactose but is produced rapidly if lactose is needed. In addition to lactose several other galactosides can induce the formation of the enzyme, one of which, methyl  $\beta$ -D-thiogalactoside, is an even better inducer than lactose although it cannot serve as a substrate for the enzyme. On the other hand, phenyl  $\beta$ -D-galactoside, which does serve as a substrate, cannot induce the synthesis of the enzyme. With either of these substances as the sole carbon source *E. coli* cannot grow. With a mixture of the two—one to serve as inducer and the other as substrate—there is good growth. This experiment is important in that it emphasizes that the inducer acts as a trigger mechanism only and does not alter the specificity of the enzyme produced.

Studies by Novick and Weiner [102] again using induced  $\beta$ -galactosidase in *E. coli* have shown that the process of induction is an all-or-none phenomenon. In a population of cells showing 50 per cent of maximum enzyme production, half of the cells are fully induced and half are not induced. This is explained on the basis of a second inducible enzyme located in the cell wall whose function is to concentrate the inducer inside the cell. This enzyme, permease, is known to exist on the basis of other studies. Normally it is present only in minute quantities if at all. If inducer is added, a cell which has some permease will begin to concentrate the inducer, which then induces more permease. Such a cell will maintain a much higher level of inducer within the cell and will remain fully induced for  $\beta$ -galactosidase in the presence of concentrations of inducer which were initially inadequate for induction.

The extent to which induction is a factor in mammalian metabolism has not been determined. A number of cases of enzyme adaptation have been reported; tryptophan peroxidase is perhaps the most exten-

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### ENZYME BLOCK

Proteins are generally considered to have two functions—structural and enzymatic. The number of enzymes known vastly exceeds the number of proteins with a recognized structural function, and the production of inherited defect is generally considered to result from the interference with the enzymatic catalysis of some specific reaction. In some cases the protein may be present but have no enzyme activity, and in others there may be no detectable protein product at all. Examples of primary enzyme deficiencies in man include phenylketonuria (Chap 10) in which the phenylalanine hydroxylase fraction I is missing [106] and galactosemia (Chap 7) in which galactose 1 phosphate uridylyl transferase is missing [106, 107].

A decrease in an enzymatically catalyzed reaction is not evidence that the specific enzyme is altered; many examples exist in which other causes can be shown. The production of an inhibitor by some other system may seriously interfere with a reaction. In phenylketonuria the interference with normal pigmentation can be best explained as an inhibition of the pigment-forming reactions by the greatly increased concentrations of phenylalanine or some of its unusual metabolites. This is readily demonstrated by the deletion of phenylalanine from the diet of such individuals; the subsequent increase in pigmentation indicates that the mechanisms for forming pigment are intact [108]. Another example is found in galactosemia in which the accumulation of galactose 1 phosphate results in a marked aminoaciduria [109] (Chap 7). Transfer to a lactose-free diet causes the amino acid excretion to become normal. In each of these examples the blocked reactions can also be deficient because of primary inherited defects in the respective enzymes.

### PRODUCTION OF PHENOTYPES

Mendel in his studies of inherited variations in peas found certain characteristics to be *dominant* over their alternatives and others to be *recessive* to their alternatives. Initially this was considered to be a property of the genetic determiner (gene). It is now apparent that the observer's interest is equally important, at least as far as attaching a label to the gene. This is illustrated very well by hemoglobin S [cf Chap 34]. From the clinical point of view, and ignoring the interaction with other rare hemoglobins, only individuals who are homozygous for hemoglobin S are affected with sickle-cell disease. This then conforms to the definition of a recessive trait. On the other hand, a person who looked only at erythrocyte preparations for the presence or absence of sickling under reducing conditions would consider the inheritance of this trait as dominant, since heterozygous individuals also show the phenomenon. A chemist looking at the composition of the hemoglobin would consider the sickles as lacking

sively studied [103] The difficulty in demonstrating induced enzyme formation in higher animals stems from the fact that only in rare cases can one study a tissue not previously exposed to substrate The differing enzyme capacities of differentiated tissues may well be due in part to differences in induction at early stages of embryonic development

Certain tissues with secretory functions may produce more or less of a protein, depending on how much has been discharged from the cell Such a circumstance can be explained by the effects of mass action, but proof is lacking on this point In other situations the amount of substrate or product is an important regulating factor An example is found in sickle cell disease where the synthesis of fetal hemoglobin is increased because of the inability of nonfetal hemoglobin to keep tissues adequately oxygenated

A potentially important aspect of the regulation of protein formation is the problem of production of nonfunctional protein at a mutant locus It is known from the many studies on hemoglobin and the recent ones of transferrin [91] that the gene products from homologous loci on different chromosomes are usually produced independently Haptoglobin is an apparent exception, but a mechanism has been suggested above which is compatible with the idea of two primary gene products If the protein itself plays a role in determining how much product shall be made then a protein which is 'non-sense' as far as particular enzyme function is concerned may be "sense" as far as regulating the amount of product is concerned The similarity of CRM to tryptophan synthetase bears out this thought Should this prove to be the case then heterozygous individuals who produce a non-sense protein may show a greater departure from normality than those who produce no protein at the mutant locus F Vogel (personal communication) has suggested this as a possible explanation for certain sex-linked genes in man For example classical hemophilia is a severe disease in *hemizygous* males (containing only one X chromosome) but shows no effect in heterozygous females Some pedigrees are known however in which heterozygous females do show minor abnormalities although the hemizygous males are less severely affected than usual [104] In such pedigrees it is not obvious why a male with one normal allele is entirely normal while a female with one normal and one abnormal shows an effect of the abnormal Interaction of the chromosomes themselves seems a most unlikely explanation If one assumes that the synthesis of protein is determined by the total amount of product formed then the normal allele may well be operating at considerably less than maximum capacity since the abnormal protein would also count as product The crucial test here would be the detection in heterozygous individuals of a nonfunctional cross-reacting product Such a mechanism may also be operating in some dominantly inherited diseases

or alteration. The analysis by Gruneberg [113] of a gene resembling achondroplasia in the rat can serve as an excellent example of the common origin of pleiotropic effects. A similar analysis of sickle cell disease in man relates the symptoms of impaired mental function, poor physical development, rheumatism, fibrosis of spleen, etc. to the single change in the hemoglobin E molecule [114]. It seems best to consider that all effects of a single gene are due to an alteration in a single primary protein, at least until clear evidence to the contrary is available.

Since mutant genes exert their effects by altering the rate of a specific chemical reaction, it is not surprising that nongenetic events can also lead to the production of a phenotype which is indistinguishable from an inherited defect. Such *phenocopies* occur primarily as developmental anomalies and probably represent differences in organization and timing of developmental processes rather than in the biochemical potential of the organism. In higher animals the relatively constant temperature and chemical environment which the mother provides for the developing embryo act as strong buffers against nongenetic aberrations, and their occurrence in man is probably more infrequent than in lower organisms. Where they are thought to occur, the biochemical mechanism for the genetic form is obscure and a comparative study at the biochemical level has not been possible. Presumably the enzyme levels would show little variation from normal. Diseases recognized as enzyme deficiencies and expressed only after birth, such as galactosemia and phenylketonuria, possibly never occur as phenocopies.

### DIFFERENTIATION

It is evident that the nuclei of germ cells contain all the chemical potential of all the cells which will subsequently differentiate into tissues of differing chemical function. The question of how certain potentials are developed and others are repressed during differentiation has long been an intriguing one; most of the answers are still to be ascertained. Since it is difficult to imagine the nucleus as gaining functions which it did not previously possess, it is generally assumed that differentiation represents a selective loss of functions or a failure to develop functions for which the necessary information was present in the original germ cell. To what extent are these losses reversible? In plants, at least, they seem to be largely reversible, as evidenced by the ability of differentiated tissues to give rise to complete plants. This ability is not widespread in animals, however, and the early embryologists had evidence that was consistent with the idea that after the first divisions of a fertilized egg, only the germinal tissues retained a complete nuclear complement.

The recent studies of King and Briggs [115] have done much to elucidate this question. They have transplanted nuclei from differentiated tissues of frog embryos at different stages of development into enucleated eggs



dominance since each allele can synthesize its specific hemoglobin regardless of the associated allele.

Phenylketonuria also provides an example in which the abnormal gene which produces disease only in the homozygous combination is detectable in heterozygous combination. Carriers of this disease have a slightly higher blood level of phenylalanine than do normal persons and they have an abnormal phenylalanine tolerance test result [110, 111]. The gene therefore is recessive as far as the disease is concerned but not as far as a change in phenylalanine metabolism is concerned.

A further point may be raised about many of the rare dominant diseases. They are generally regarded as situations in which the presence of only one abnormal gene is sufficient for expression of the disease. Actually, because of the rarity of these genes, it is seldom known what their expression would be in the homozygous combination.

The terms *dominant* and *recessive* will continue to be useful descriptions of the expressions of different alleles. In using them one should remember that they are related to a particular type of observation and that particular findings result from the interaction of the gene with other systems.

Variations of other systems, both genetic and nongenetic, may influence considerably the intensity of expression of a particular gene, in some cases enhancing its effects and in others deleting its effects entirely. In working with nonpurebred strains such as man, the variation in genetic expression is apt to be particularly evident. In many cases a mutant trait will not be expressed at all in an individual who is shown to have the mutant genotype by pedigree analysis. Genes which are always detectable if present in the correct combination (heterozygous for dominants, homozygous for recessives) are said to be completely *penetrant*. Genes which do not always produce the detectable end result are said to have lowered penetrance. Although penetrance can sometimes be used to distinguish between two mutant genes having the same end result, the level of penetrance will vary with the level of observation, just as in dominant versus recessive traits. Thus a child with genes for galactosemia will not develop the disease on a lactose-free diet [112]. If such diets were prevalent then the penetrance of galactosemia would be much lower than it in fact is. On the other hand, galactosemic individuals reared on a lactose-free diet still have an abnormal galactose tolerance test result, and if such a test were the criterion for the trait, then the penetrance would be complete.

Many genes produce a variety of multiple effects involving functions not otherwise known to be related. The term *pleiotropism* has been used to describe this phenomenon. It is difficult to reconcile a true multiple gene action with the one gene—one enzyme theory discussed earlier, and in those cases of pleiotropism which have been adequately analyzed, all the observed changes can be traced ultimately to a single primary defect.

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and have then studied the ability of these eggs to develop into embryos. Their experiments clearly demonstrate that nuclei from early embryos can give rise to normal embryos when transplanted. The older the donor embryo, the more abnormalities were observed. Even if the transplanted nuclei were obtained from the late gastrula stage, a few normal embryos were obtained. Thus the genetic information had remained intact in at least some of the cells through this stage of development. It was also noted, however, that nuclei transplanted from a particular donor tissue tended to promote development of that tissue in the recipient egg, suggesting that some type of nuclear differentiation possibly had occurred.

Another example of the role of genotype in differentiation is found in the work of Stern [116] using *Drosophila*. It is possible to obtain genetic mosaics in *Drosophila* in which parts of the tissues of an individual differ by one or more genes from the remainder of the fly. Events which occur at some specific place, such as the formation of a bristle, may be inhibited by a genotype for absence of bristles at that place. If tissue containing normal genotype is not too distant, then the bristle may form at the site of this normal tissue, a place where it would never form in a normal fly. Thus the genetic potential for bristle formation is retained by tissue which is not required to form bristles during normal development.

Such studies neither prove nor disprove the hypothesis that the genetic information remains intact in all the cells of a fully differentiated animal. They do prove that factors other than selective genetic loss are responsible for the early stages of differentiation and suggest that such factors, by their ability to inhibit the function of certain genes, are responsible for all differentiation.

## MODES OF INHERITANCE—ANALYSIS OF PEDIGREES

The operational nature of such terms as dominance, recessiveness, and penetrance has been pointed out. Nevertheless, these terms have a very real meaning in the prediction of the recurrence of inherited traits. Such predictions are commonly used for two purposes—in genetic counseling and in the understanding of genetic mechanisms. The discussions in this section will be concerned with the latter.

The analysis of pedigrees can be a potent tool in the recognition of identity or nonidentity of mutant genes. One example is in the differentiation of two genes on the basis of their linkage groups. In man, this normally consists in recognition that one of the genes is transmitted on the X chromosome while the other is transmitted on an autosome. This distinction has been useful in recognizing different forms of muscular dystrophy and some of the blood clotting disorders. A single example exists in which autosomal linkage has been used to distinguish two abnormal genes otherwise similar in their manifestations. This is the

case of elliptocytosis linked to the Rh blood type in some but not all pedigrees [14]. It is to be expected that as more markers become available for human chromosomes this approach will become much more powerful than it is at present.

A second case in which it is possible to recognize the existence of more than one form of abnormal gene is that in which the forms show different epistatic relationships (i.e. dominance or recessiveness). This is illustrated by the work of Harris, Mittwoch, Robson and Warren on cystinuria [117]. In one form of the disease the carriers show no abnormality in their urinary excretion of cystine and lysine [Chap. 42]. In the other form the carriers can be detected by their increased excretion of cystine and lysine. Thus one type is recessive and the other incompletely recessive. The homozygous individuals are similar in the two forms of the disease. This evidence that two types of abnormal gene exist does not tell us whether these two types are allelic or not. Thus these two forms of cystinuria may represent mutations of two functional genetic loci located on different chromosomes or they may be alleles, one of which is slightly more deleterious in its action.

Families in which more than one recognizable gene is segregating can sometimes yield much information on genetic mechanisms. Thus one parent cannot pass two alleles to the same offspring. If two genes are passed from parent to offspring then this is sufficient evidence for their being nonallelic. Again nonallelism is indicated if neither gene is transmitted to an offspring. This is illustrated by the family already described in which hemoglobins  $\beta^E$  and  $\beta^G$  were shown to be nonallelic. In such critical cases it is obviously important to establish correctly the family relationships.

In the remainder of this chapter are reviewed some of the simple forms of inheritance.

### CHROMOSOMAL INHERITANCE

Virtually all recognized genetic variation is Mendelian in that it involves genetic determiners located on the chromosomes. These determiners are transmitted to the offspring in a regular manner as outlined at the beginning of this chapter, resulting in only a few well-defined modes of inheritance. These different modes are caused by differences in dominance versus recessiveness and in autosomal versus sex linkage. The common patterns of inheritance are outlined in the following sections.

#### *Autosomal Dominant Inheritance*

A gene which is fully expressed when in the heterozygous combination is said to be dominant, and if it occurs on other than the sex chromosomes it is said to be autosomal. At least one parent of any affected individual will also carry the gene, and if the gene has high penetrance that parent

will also be affected. A gene may be expressed in only one sex but if it is an autosomal dominant, then it will be *transmitted* by parents of either sex to children of either sex. If the gene is rare then usually only one parent will be affected. Approximately half the children of an affected person will also be affected. Occasionally a mutation may occur giving

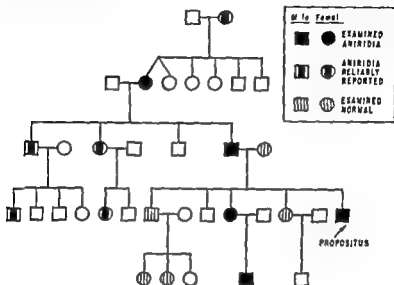


Fig 2 11 A pedigree of an individual illustrating autosomal dominant pattern of inheritance

rise to an affected individual from unaffected parents. A pedigree of a simple dominant trait is shown in Fig 2 11.

### Autosomal Recessive Inheritance

If a trait is expressed only in the homozygous combination then it is recessive. This common mode of inheritance for diseases of enzymatic defect is characterized as follows: (1) For a rare gene the affected individuals in a family will nearly always be in a single sibship. (2) For a rare gene the parents of an affected individual will be relatives more often than in the general population. (3) Approximately one fourth of the siblings of an affected individual will also be affected. (4) Homozygous males and females should occur in equal numbers although the expression may vary greatly with sex. A pedigree of a simple recessive trait is shown in Fig 2 12.

### Sex linked Dominant Inheritance

Conventionally a mutant gene is thought to have a decreased ability to carry out a function. Since a normal male has only one X chromosome a female who has only one mutant gene on an X chromosome has the

same number of normal genes as a male and should be able to function as well. Using this line of argument sex linked dominants should not exist at all. They are in fact exceedingly rare. A discussion of their possible biochemical mechanism has already been given (see Regulation of Protein Synthesis above). For such a model of inheritance, (1) affected males will always have an affected mother while affected females will have an affected father one third of the time and an affected mother two thirds of the time. (2) The daughters of an affected male will all be

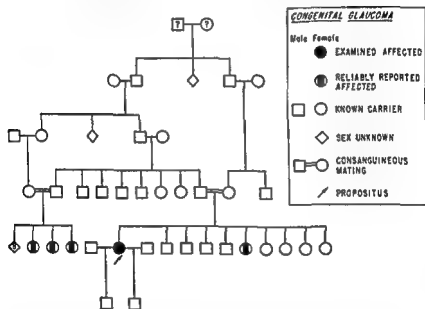


Fig. 12. A pedigree of congenital glaucoma illustrating autosomal recessive pattern of inheritance. The individuals marked as carriers are known to be such because of their relationship to others in the pedigree. Some of the individuals not marked are undoubtedly carriers but this cannot be demonstrated.

affected while none of the sons will be affected. (3) An affected female will transmit the trait to half her sons and half her daughters. (4) Since females have twice as many X chromosomes as do males, there will be twice as many affected females in the population as there are males. An example of sex linked dominant inheritance is found in Fig. 6-7.

### Sex linked Recessive Inheritance

This mode of inheritance has long been recognized in man because of several unique properties. In males, a single mutant gene will be expressed since there is no normal allele to function. In females, on the

other hand the mutant gene must be present on both X chromosomes in order for the trait to be expressed. Thus (1) affected males will *never* transmit the trait to their sons but all their daughters will be heterozygous "carriers." (2) Heterozygous females will transmit the mutant gene to half their sons who will be affected and to half their daughters who will be carriers. (3) If an affected male marries a carrier female then half their offspring will be affected regardless of sex. (4) The sons of affected females will always be affected. (5) For sex linked recessive traits more males will be affected than females. The rarer the gene, the higher the ratio of males to females.

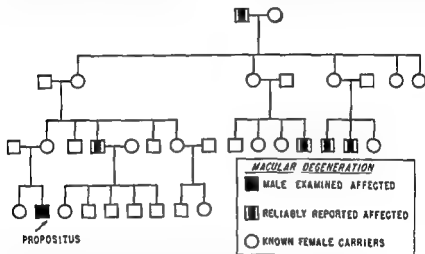


Fig. 2-13. A pedigree of macular degeneration illustrating sex linked recessive pattern of inheritance. The females marked as carriers are known to be such because of their relationship to affected males. Some of the other females may also be carriers.

An example of a pedigree showing sex linked recessive inheritance is given in Fig. 2-13. It is necessary to distinguish between sex linked inheritance and sex limited inheritance. In the latter case the trait is expressed only in one sex but the pattern of inheritance is that of an autosomal gene. Such a gene can be transmitted from father to son a mode never found in sex linked inheritance.

### *Inheritance on the Y Chromosome*

The Y chromosome has been envisioned as consisting of two segments—one homologous with a portion of the X chromosome and the other non homologous with the X chromosome. The sex linked genes already discussed would occur on the nonhomologous portion of the X chromosome. Genes occurring on the homologous portions of the sex chromosomes are referred to as "partially sex linked" and genes occurring on the non homologous portion of the Y chromosome are referred to as "holandric."

or Y linked Haldane [118] first suggested the existence of partial sex linkage in man but subsequent attempts to verify it have been unsuccessful and it is now considered doubtful that there exists a homologous segment at all

There are a number of pedigrees in the literature for which holandric inheritance is the most probable explanation Stern [12] in a masterful piece of detective work has shown the need for revision of some of the more striking of these pedigrees with the result that they become more compatible with other modes of inheritance Although he is duly cautious about excluding the possibility of complete Y linkage he very effectively points out the inadequacy of the evidence for its existence In complete Y linkage affected males will transmit the trait to all their sons and to none of their daughters It is necessary to distinguish this mode of inheritance from an autosomal dominant trait which is expressed only in males In the latter case half the daughters of an affected male though unaffected themselves would be able to transmit the trait to their sons

#### CYTOPLASMIC INHERITANCE

The long-debated question of the presence of genetic determiners in the cytoplasm has in recent years been resolved in their favor [119-120] The demonstration of such a phenomenon rests on the premise that spermatozoa carry with them virtually no cytoplasm and hence cannot transmit the trait in question while eggs from a particular individual will all carry the same information in their cytoplasm Thus all the progeny would resemble the female parent and none the male parent in a mating between two individuals differing in some cytoplasmic factor This expectation is quite unlike any for traits whose variation is controlled by the nucleus

One of the more striking demonstrations of cytoplasmic inheritance occurs in paramecia [121] Certain of these organisms elaborate a substance into the medium which is toxic for other individuals The former killer paramecia were shown to transmit the trait through their cytoplasm and the trait has been shown to be due to the presence of particles in the cytoplasm of killer cell These particles are about 0.4 micron in diameter and they stain with the Feulgen stain which indicates their deoxyribonucleic acid nature They are capable of replicating and being transmitted by the cytoplasm Their maintenance however requires the presence in the nuclear genome of a particular dominant gene  $k$  and they cannot be transmitted to cells which lack  $k$  Cells which lack the  $k$  particles in their cytoplasm are sensitive to its toxic effects regardless of their genotype

Other examples of cytoplasmic inheritance have been found in *Neurospora* of which the *poly* strain was the first observed [122] This strain is characterized by very slow rate of growth although in a contained cul-



other hand the mutant gene must be present on both X chromosomes in order for the trait to be expressed. Thus (1) affected males will *never* transmit the trait to their sons but all their daughters will be heterozygous "carriers." (2) Heterozygous females will transmit the mutant gene to half their sons who will be affected and to half their daughters who will be carriers. (3) If an affected male marries a carrier female then half their offspring will be affected regardless of sex. (4) The sons of affected females will always be affected. (5) For sex linked recessive traits more males will be affected than females, the rarer the gene the higher the ratio of males to females.

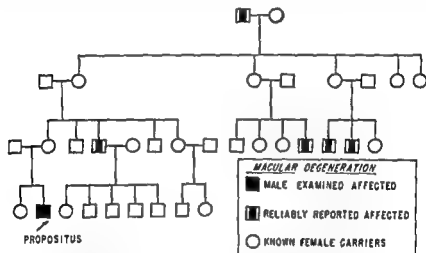


Fig. 13. A pedigree of macular degeneration illustrating sex-linked recessive pattern of inheritance. The females marked as carriers are known to be such because of their relationship to affected males. Some of the other females may also be carriers.

An example of a pedigree showing sex-linked recessive inheritance is given in Fig. 213. It is necessary to distinguish between sex-linked inheritance and *ex* limited inheritance. In the latter case the trait is expressed only in one sex but the pattern of inheritance is that of an autosomal gene. Such a gene can be transmitted from father to son in a mode never found in sex-linked inheritance.

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Other examples of cytoplasmic inheritance have been found in *Neurospora* of which the *poly* strain was the first observed [122] This strain is characterized by very slow rate of growth although in a contained cul

ture the final growth approaches that of the wild type. A mating of poky female (protoplasticum) by wild type male (conidia) produces only poky offspring while the reciprocal mating produces only wild type offspring. The trait was found to be caused by the presence of abnormal mitochondria which among other differences, have an unusual cytochrome composition.

Mitochondria, as well as other particles replicate in the cytoplasm and are then distributed randomly when a cell divides. The demonstration of a transmitted variation influenced by the nucleus raises questions as to the nature of the role which the nucleus plays. Does it have primarily a "permissive" function, with most of the genetic specificity residing in the multitude of cytoplasmic particles or does it actually supply most of the detailed information necessary for the many synthetic processes of the cell with the variation in cytoplasmic factors occurring only as a rare event? The evidence favors the latter view. First cytoplasmic inheritance has not been often detected and has never been demonstrated in higher animals. Second the detailed amino acid composition of proteins such as hemoglobin is controlled by factors which segregate in a Mendelian fashion. It seems best to regard cytoplasmic inheritance as an infrequent occurrence particularly in man but one which must be considered in the genetic analysis of variation.

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## Part Two

### Diseases Manifest Primarily as Disorders of Carbohydrate Metabolism

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## Chapter 3

### Diabetes Mellitus\*

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*Albert L. Renold and George F. Cahill Jr*

Although there is little reason to doubt that future developments will fully justify the decision of the editors to consider diabetes mellitus an inborn error of metabolism there is equally little reason to deny that the nature of this error is at present shrouded in darkness. Why then discuss this ancient disorder in a text whose title implies some degree of knowledge about the nature of the inherited metabolic aberrations to be discussed? Justification may perhaps be derived from at least three considerations.

1 Diabetes mellitus is a widespread and complex disorder of metabolism intimately related in history to the development of understanding of the metabolism of carbohydrate, protein and fat and thus well suited for the illustration of over all schemes of intermediary metabolism and of their interrelations. Its historical importance is compounded by its practical importance which derives from its high level of incidence and its prevalence among all human races and societies.

2 Diabetes mellitus—more precisely the susceptibility to diabetes mellitus—is indeed conditioned by genetic factors and may thus quite properly be termed inborn.

3 A discussion of the knowledge accumulated about this disorder may serve to illustrate how much ingenious and exhaustive effort can be devoted to the study of a metabolic disorder without even approaching the nature of the inborn disorder. This last consideration is possibly more important than would first appear since it may be useful in tempering excessive enthusiasm with regard to the final nature of the inborn metabolic errors to be described in other sections of this volume. Although without question *closer* to the primary defect some of these may also

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of moderate hyperglycemia alone. The causal relationships assumed in the sequence of events just described are convenient and likely but in many aspects still hypothetical. More will be said on this subject below during consideration of the metabolic effects of insulin.

### *Chronic*

The term *chronic diabetic syndrome* refers to the many clinical manifestations which in addition to some or all of the manifestations of the acute diabetic syndrome are associated with the presence of diabetes of long duration. These manifestations may affect any system or organ although the organs most frequently affected are the kidneys, eyes, heart, muscle, and portions of the nervous system including the peripheral nerves. A large part of these manifestations appears to have a vascular basis, sometimes interpreted as accelerated vascular aging although more specific alterations of capillaries, arterioles, and venules are frequently present, particularly in the eyes and kidneys. The frequent presence of abnormal deposits of glycogen and mucopolysaccharides in diabetic cells in general and in the basement membrane of capillaries and small blood vessels in particular has led to the suggestion that a metabolic aberration of small vessel walls might be at the origin of many pathologic and clinical manifestations of the chronic diabetic syndrome [6-8]. Whether the acute and the chronic diabetic syndromes are directly related causally is as yet uncertain although likely in the opinion of many.

Most of the following discussion will be limited to the acute diabetic syndrome. Whether or not the manifestations of the chronic syndrome are related to an inborn error of metabolism and if so whether or not the error is that responsible for the acute syndrome are as yet so hypothetical as to preclude useful discussion in this text. The clinical differentiation of two or more diabetic syndromes (e.g. growth-onset versus adult diabetes) will be briefly discussed below under Insulin.

## GENETIC CONSIDERATIONS

### *Incidence*

The over all incidence of diabetes mellitus is difficult to ascertain since the disease may declare itself late in life and may go unrecognized for many years. The most accurate attempt to evaluate this incidence stems from the studies of Wilkerson and Krall who in 1947 tested 3516 of 4983 inhabitants of a New England community with an age distribution closely approximating that of the United States as a whole [9]. They found 40 known and 30 previously unknown diabetic patients, i.e. a prevalence of 2.0 per cent of those tested or a projected 1.7 per cent of the entire population of the town. Follow up studies indicate that the



prove to be but *manifestations* of the primary defect separated from it by one or several intermediate metabolic or structural conductors

Published evidence concerning every aspect of diabetes mellitus is so extensive as to preclude any attempt to provide complete coverage of possible topics, or of any single topic Accordingly the authors have, *tolens nolens*, exercised arbitrary selectiveness with emphasis upon concepts and upon attempted synthesis and organization rather than upon erudition and documentation Major omissions will be noted by all and these the authors regret although without apology

Rather than providing an abbreviated historical introduction, the authors refer to the remarkable monographs of Allen [1, 2] for the pre-insulin period and to the monograph of Jensen [3] for the period which led to the discovery and application of insulin

## DIABETIC SYNDROMES

Although Dobson [4] concluded from his experiments published in 1776 that the saccharine matter secreted by the kidney in diabetes was "not formed by the secretory organ but previously existed in the serum of the blood," Ambrosiani is credited with having first recorded in 1835 that the blood of diabetic patients contains more sugar than that of nondiabetic individuals [5] and thus with having established what is now accepted as the only essential sign of the disorder i.e. *hyperglycemia* The severity and duration of hyperglycemia (with or without provocation) required to establish the diagnosis of the disease vary from medical center to medical center a widely accepted set of criteria is that established by Joslin and his group [5]

### *Acute*

Under the term *acute diabetic syndrome* are grouped all those manifestations more or less readily explained as derived from the cardinal manifestation—hyperglycemia With increasing hyperglycemia, after the renal tubular capacity for glucose reabsorption is exceeded glycosuria and with it, polyuria result Loss of water and glucose leads to increased thirst and hunger Glucose loss by draining the carbohydrate supplies of the organism results in increased need for mobilization and catabolism of proteins and fats This results in weight loss and when the mobilization of fats becomes excessive in the accumulation of ketone bodies in the blood and urine, with ketonuria and ketonemia Since the ketone bodies are primarily anionic cations accompany their urinary excretion When the blood levels of ketone bodies become excessive ketoacidosis and by as yet unclear mechanisms coma and death result The sequence is frequently rather rapid (several hours to several days) although it may remain transiently or permanently arrested at any stage even at that

*Inheritance Pattern*

The opinion most prevalent today assigns to the predisposition for diabetes mellitus a simple Mendelian recessive mode of inheritance [20] ✓ This is first and rather directly suggested by the tendency for the disorder to skip generations a repeatedly documented observation accessible to any thoughtful questioner Second many comparisons between observed and predicted incidence of diabetes in specific areas or groups have yielded best agreement when the predicted figures were based on the assumption of a simple Mendelian type of inheritance Furthermore a number of very extensive and well-documented genealogies can be interpreted on this basis only In the population group studied by Hanhart and referred to above [16] the incidence of diabetes among the 1 623 siblings of the 304 diabetic subjects was 27.3 per cent whereas it was 22.0 per cent in their 646 offsprings both figures being corrected statistically for the age behavior of diabetes There was thus a significantly greater incidence of diabetes among the siblings than among the children of diabetic persons a pattern which can be explained only on the basis of recessive inheritance The significance of this observation is further increased by comparing this incidence with that found among 114 children aged 40 or more of 35 instances of conjugal diabetes (both parents having known diabetes) also studied by Hanhart [16] and drawn from the same general population When corrected for the age behavior of diabetes the incidence obtained in this group was 94 per cent!

The arguments brought forward in the preceding paragraph strongly suggest the participation of a simple Mendelian recessive trait in the inheritance of the potentiality for developing diabetes they in no way however establish a monopoly for such a trait Genealogies singly or in small numbers strongly suggesting simple dominant [21-22] or sex linked recessive inheritance [23] have been reported The occurrence of diabetes in three successive generations is not really rare while its occurrence in four successive generations has been recorded twice in the experience of the Joslin Clinic [13] However it is evident that the combination of a very high incidence of diabetes at least in certain areas (see first paragraph) with the prolonged latency characteristic of this disorder may lead to any number of freak genealogies resulting from inability to recognize either the heterozygous carrier or frequently the homozygous latent diabetic In many instances the pseudodominant nature of what first appeared to be a dominant pattern has been established by extension of the genealogy or by the passage of time

*Penetrance*

In order to explain the absence of diabetes in a significant percentage of identical twin mates of diabetic twins (Table 3.1) as well as the fact that

incidence of the disease in the original population if followed to old age or death will be at the very least double that of the prevalence initially found i.e., 3.5 per cent or more likely higher [10, 11]. Although these figures are higher than those generally accepted [11a] this may primarily be the result of the adequacy of the procedures used for the detection of abnormal glucose metabolism in this study. These figures are mentioned here since acceptance of a high rate of incidence of diabetes mellitus (or rather of the tendency to develop the disorder) is necessary in order to explain some of the genetic irregularities to be discussed below.

### *Evidence for Hereditary Nature of Diabetic Potential*

That the potentiality to develop diabetes mellitus is inherited has been surmised for many centuries [12] and is now an established fact. This rests primarily upon (1) the greater frequency of diabetic persons among close relatives of known diabetic patients than among suitable control populations and (2) the even more frequent occurrence of diabetes in

TABLE 3.1 INCIDENCE OF DIABETES IN TWO SERIES OF TWINS

| Author     | Similar twins |                          |          | Dissimilar twins |                          |          |
|------------|---------------|--------------------------|----------|------------------|--------------------------|----------|
|            | Total No      | Diabetes present in both |          | Total No         | Diabetes present in both |          |
|            |               | No                       | Per cent |                  | No                       | Per cent |
| Then Bergh | 16            | 30                       | 6.2      | 87               | 18                       | 2.1      |
| White      | 33            | 16                       | 48       | 63               | 2                        | 3        |
| Total      | 49            | 46                       | 58       | 150              | 20                       | 13       |

the mates of similar diabetic twins. With regard to the first point only one study will be mentioned here among many [13]. Hanhart [14-16] has analyzed 304 genealogies of diabetic patients of the Joslin Clinic with 18,493 members and compared them with 110 genealogies of nondiabetic individuals with 6,042 members. Both groups were drawn from a socially and racially similar population. Among the 18,493 members of the diabetic genealogies 975 diabetic persons were found, an incidence of 5.3 per cent, whereas only 74 diabetic persons or 1.2 per cent were found among the 6,042 members of the nondiabetic families. The incidence of diabetes in two series of twins [17, 18] is shown in Table 3.1. Differences between the two series are primarily the result of the criteria used for establishing presence or absence of the disorder in the twin mates of diabetic twins. It is difficult to avoid the conclusion, however, that whatever the true incidence, the incidence of the disease in identical twin mates grossly exceeds the incidence which may be expected in any general population group.

of the metabolic decompensation process accompanying pregnancy may become permanent [32] The reported increased incidence of diabetes in multiparous women over 40 years of age (when compared with men or with women not having borne children) supports this contention [35] This relation between parity and the development of diabetes after the age of 40 however, is not universally accepted [26]

### *Etiologically Distinct Forms of Diabetes*

The variable clinical manifestations of diabetes mellitus in man have frequently led to the postulate of etiologically distinct forms of the disorder This concept would be strengthened were it possible to demonstrate at least some degree of genetic separation of these clinically distinct syndromes Generally it is accepted today that such a genetic separation has not been demonstrated [5, 16 20 36 37] and that, for instance growth-onset type and adult onset type diabetes frequently occur within the same family A few opinions to the contrary have been recorded [38 39] The widespread prevalence of the diabetic trait (or traits) in the general population greatly adds to the difficulty of establishing the presence or absence of genetic separation of different diabetic syndromes particularly in highly mobile and mixed population groups

It is of interest therefore to find that although there is a slightly higher incidence of diabetes among Jews and although it has been repeatedly stated that this might be the result of the somewhat greater degree of inbreeding of this population diabetes mellitus among Jews does not appear to have assumed any characteristic clinical pattern

### *Intecedence and Anticipation*

The terms antecedence and anticipation in the inheritance of diabetes mellitus were used to describe the tendency for the overt disease to occur earlier in life in succeeding generations Fortunately this concept for which it was difficult to find a reasonable genetic explanation has been shown to represent most probably a statistic rather than genetic phenomenon [40] it should dissolve with the passage of time and the recording of genealogies with adequate follow up periods

At the beginning of this section it was stated that the most intensive study designed to estimate the true incidence of diabetes in the United States indicated a value of 3.5 per cent or more Assuming that individuals liable to diabetes are homozygous for a recessive gene ( $dd$ ) and basing their estimate upon two different sets of data from this country Steinberg and Wilder [20 36 37 41 42] concluded that approximately 5 per cent of the population of the United States are homozygous ( $dd$ ) for the gene determining susceptibility to diabetes These estimates clearly indicate the enormous practical importance of this disorder

the incidence of diabetes among the offspring of marriages between diabetic patients usually lies far below 100 per cent and frequently remains below 100 per cent even when correction for the age behavior of diabetes is made it is customary to state that *the penetrance (i.e., likelihood that a gene will receive its morphologic or functional expression) of the diabetic trait is incomplete and furthermore variable*. This incomplete penetrance could result from the dependance for expression upon either associated genetic factors or associated environmental factors. Since the genetic capital of identical twins should be identical the importance of associated environmental factors appears more probable [19-20]. Furthermore Hanhart [15] has studied seven genealogies of conjugal diabetes in which considerable and long standing inbreeding existed thereby increasing the likelihood of the genetic homogeneity of the diabetic trait. In all seven families at least one child was diabetic but in no instance were all of them, again suggesting that the variable penetrance of the trait is due to nongenetic factors. It is of interest to note that this type of evidence rather clearly suggesting the importance of environmental factors upon the incidence of the disorder within genetically equally jeopardized offsprings offers perhaps the most hopeful basis for eventual prevention of at least overt diabetes.

### *Pregnancy*

Among the environmental factors which have been considered particular importance may attach to the *environment of the fetus in utero*. Since diabetic mothers have been enabled to carry pregnancies to term only relatively recently statistically fully adequate comparisons of the incidence of diabetes in the offsprings of diabetic mothers and in the offsprings of diabetic fathers have not as yet been made a greater early incidence in the former has been suggested [24-25] but also questioned [26] while the incidence of large babies is very likely greater when the mother was the diabetic or prediabetic partner [27]. Even mild and unsuspected diabetes during pregnancy (see Prediabetic State below) greatly increases fetal wastage and the pathologic anatomic aberrations found include an abnormal appearance of the fetal islets of Langerhans suggesting hypertrophy [28-29]. Accordingly it would not be surprising if time were to substantiate the claim that the metabolic state of the mother during gestation may influence the incidence of overt diabetes in the offspring or at least the timing of its onset. Experimental models in animals have been suggested [30, 31] and may well lead to an earlier answer to this important question.

Whereas maternal metabolism must be of importance to the fetus it is also well established that the metabolic changes of *pregnancy* (for the mother) frequently uncover the existence of a previously latent diabetic trait [32-34]. It has been strongly suggested that the acceleration

diagnosis can be made by methods of testing accepted at the time of their performance. With increasing ability to detect the homozygous (dd) carrier of the diabetic trait prediabetes should become an even shorter period of the life of each diabetic patient. This is desirable since in the majority of instances early recognition of the presence of homozygosity for diabetes will enable the physician to study and perhaps to influence that important and often extensive early period of the disease which is not accompanied by symptoms but which undoubtedly plays a major role in the timing and nature of its clinical course.

In order to detect an even greater number of homozygotes at an even earlier time in their life tests have been devised which study the adequacy of glucose metabolism under conditions of stress. This stress may be the administration of a glucose load or it may combine glucose loading with other stresses such as the administration of cortisone or the coexistence of pregnancy. To date the most extensive and successful effort made to detect a large proportion of prediabetic individuals in a given population has been the study by Conn [51-51a] of the relatives of diabetic patients followed by his unit at the University of Michigan. Among 387 non-diabetic close relatives of known diabetics performance of glucose tolerance tests revealed 71 hitherto unknown diabetics (18 per cent) and performance of a combined cortisone glucose tolerance test in 259 of the subjects of this group which had shown a normal response to glucose alone uncovered another 64 individuals whose response was abnormal by the criteria used (16 per cent of the original group). In contrast an abnormal response to glucose alone was observed in only 1 and to cortisone and glucose in 3 of 125 individuals without known diabetic relatives (1 and 3 per cent respectively). Thus in this study testing of 387 close relatives of known diabetics all previously considered unaffected demonstrated that upward of 34 per cent should be considered diabetic by the criteria used.

Other investigators [24-32-33] among whom Hoet is particularly active have suggested that advantage should be taken of the physiologic stress of pregnancy to detect the presence of prediabetes in mothers. The detection of prediabetic persons in this group may be of special importance both to the mother and to the child and careful therapy of any degree of carbohydrate intolerance during pregnancy has been strongly recommended. In the absence of glucose tolerance studies during pregnancy the repeated observation of unusually large babies and particularly progressive increase in infant weight during successive pregnancies suggests maternal prediabetes [52].

Other stressful situations frequently associated with decreased carbohydrate tolerance are puberty, obesity and infection. Since decreased carbohydrate tolerance in these situations is often temporary only its documentation is of great importance in order to establish prediabetes. The frequent association of decreased carbohydrate tolerance and obesity

particularly since the introduction of effective therapy is likely to result in a further increase of this incidence

### *Hereditary Diabetes Mellitus in Animals*

Although the occurrence of spontaneous syndromes resembling human acute diabetes mellitus has been noted in many species the authors are aware of the existence of only two fully documented hereditary diabetic syndromes in animals. In 1949 Ingalls et al [43] reported the occurrence of a mutation in mice of the Jackson Memorial Laboratory Bar Harbor, Maine, a mutation resulting in the simultaneous appearance of *obesity and hyperglycemia*. The trait is recessive both obesity and hyperglycemia appear to be linked to a single gene. The pancreatic islets of these mice are hypertrophic and increased insulin like activity has been found both in pancreatic extracts [44] and in blood serum [45] of the obese-hyperglycemic animals when compared with normoglycemic nonobese litter mates. The affected mice do not develop ketosis [46]. In 1950, Meier and Yerganian reported occurrence of diabetes mellitus during inbreeding of several lines of Chinese hamsters (*Cricetus griseus*) [47-48]. In contrast to the obese-hyperglycemic mice diabetic Chinese hamsters exhibit degenerative changes of the islets of Langerhans and tendency to profuse glucosuria and ketonacidosis. Liver slices from diabetic animals demonstrate a metabolic derangement quite comparable to that seen in rats with severe alloxan diabetes [49]. The possible occurrence of some features of the chronic diabetic syndrome in these animals has been reported in preliminary form [48]. If laboratory animals with hereditary forms of diabetes mellitus should become available it is evident that this might well be one of the most significant forward steps in understanding the genetic and environmental factors concerned with diabetes mellitus in man.

It may be of interest to recall at this point that sensitivity to insulin is also subject to genetic control. Not only do different species vary greatly with regard to insulin responsiveness but within a given species unusually sensitive and unusually resistant strains have been described. Thus the K.L. strain of mice may be given intraperitoneally from 300 to 500 units of insulin with impunity whereas their controls regularly convulse and succumb after the injection of doses well below 50 units [50].

### *Prediabetic State*

If the hereditary nature of diabetes mellitus is accepted and since it is known that the overt disease may not develop until late in life it is evident that a large number of individuals who are genetically destined to become diabetic are considered to be totally unaffected for significant periods of their life. As defined by Conn and Lajans [51-51a] the 'prediabetic state' must be regarded as existing in an individual destined to become diabetic from the time of conception to the time when a definitive

guinea pigs and has recently been confirmed by Wright [58] in rabbits injected with antibodies to beef insulin obtained in guinea pigs (Fig 3 1) As would be anticipated this type of diabetes is reversible with discontinuation of the administration of these species specific insulin antibodies

It would seem almost impossible to produce a better biologic correlation between the experimental production of the diabetic syndrome on the one hand and interference with the endocrine function of the pancreas on the other If in addition one considers that the administration of insulin is capable of completely reversing all known features of the acute diabetic syndrome the weight of evidence seems almost overwhelming There are however, additional experimental diabetic syndromes in animals in which the relationship to the insulin producing cells of the pancreas is less evident Among these are (4) the prolonged administration of anterior pituitary extracts [59] or of purified pituitary growth hormone [60] preparations as well as in suitably prepared animals of certain adrenocortical [61] and thyroid hormones [62] It has been postulated that the metabolic effects of these hormones create conditions leading to persistently increased insulin secretion [63] and thereby to eventual inadequacy of the insulin secreting cells A similar mechanism has been postulated for the production of a diabetic syndrome by (5) the prolonged administration of glucose in quantities sufficient to maintain a greatly elevated blood glucose level throughout the greater portion of the 24 hr period [64] This type of experimental diabetes has been reproducibly obtained in only one species the cat

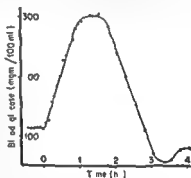


Fig 3 1 Concentration of glucose in the blood of a conscious female rabbit (♀ 60 kg) following the intravenous injection at 0 time of serum (2 7 ml) from guinea pigs sensitized to bovine insulin (From Wright [58])

### Human Diabetes

Human diabetes mellitus has been produced by pancreatectomy incident to removal of malignant tumors and has been noted during and after pancreatitis In addition hemochromatosis which is accompanied by a deposition of iron pigment in the pancreas with secondary fibrosis and loss of functioning tissue including particularly islet cells is a well established cause of diabetes mellitus In these instances all relatively rare some correlation exists between the development of diabetes and destruction of the pancreatic islets In the great majority of cases of spontaneous diabetes mellitus in man however the correlation between



has sometimes been ascribed to a genetic rather than environmental link. It is tempting to believe that this may be true in certain instances (particularly when one considers the existence of the obese hyperglycemic syndrome in mice), but at present it does not seem feasible to dissociate genetic and environmental factors in any of the studies reported.

The great importance of prediabetes and of developing methods suitable for its detection is based on at least three major considerations: (1) ability to detect homozygotes (dd) for the diabetic trait would place genetic studies of diabetes on a sounder basis, thereby opening the way for sounder genetic advice, wherever acceptable; (2) it would seem evidently desirable to gain insight into the *true* incidence of the diabetic trait in order to uncover some of the interrelations between diabetes and the several major disorders of cardiovascular renal origin which afflict civilized man; (3) most importantly, if large groups of individuals were known to be the as yet asymptomatic carriers of the homozygous trait, investigative programs designed to establish some of the environmental conditions needed to precipitate or prevent the onset of the overt phase of the disorder could be conceived with at least some degree of intelligence and chance of success.

## ENDOCRINE DEFECTS IN DIABETES

### *Experimental Diabetes*

The major endocrine defect in diabetes mellitus is considered to be absolute or relative insulin deficiency. It should be pointed out at the outset that this does not necessarily imply a primarily defective insulin-producing tissue.

Understanding of the proper nature of the major endocrine defect is primarily based upon observations concerning the production of *experimental diabetes* and goes back to the demonstration by von Mehring and Minkowsky in 1889 that removal of the pancreas in dogs produces a syndrome grossly similar to the acute diabetic syndrome in man [53]. As of the present, experimental diabetes mellitus can be produced by one of the following procedures: (1) the surgical removal of all or at least a large portion of the pancreas; (2) the chemical destruction of all or at least a large portion of the beta cells of the islets of Langerhans — destruction which can be achieved with substances such as alloxan [54], dehydroascorbic acid [55] or dithizone [56] which in appropriate doses produce a selective necrosis of the beta cells; (3) the inactivation of circulating insulin by the administration to animals of one species of antibodies effective against its own insulin and obtained by immunizing other animals. This type of diabetic syndrome was first described by Moloney [57] in mice injected with antibodies to pig insulin obtained in

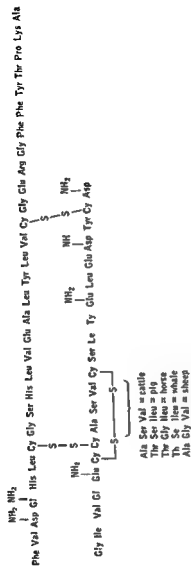


Fig 3- Chemical formula of insulin (Modified from Sanger [68] )

morphologic damage to the islets and the development and severity of the disease is not good, but it is not so poor as has been frequently stated.

Whereas it is true as recently restated by Warren and LeCompte [8] that from one-fourth to one-half of all cases of diabetes show no changes in the pancreas extensive enough to constitute a convincing cause of insulin deficiency these authors also point out that this is true only when one considers the material obtained and *studied histologically by routine methods*. When special attention is paid to the insulin producing beta cells and to the tedious and time-consuming estimation of total mass of pancreatic beta cells, a decreased volume or abnormal appearance of the beta cells is almost uniformly found [65-66]. This anomaly is most marked in patients with growth onset type of diabetes in whom the acute diabetic syndrome if untreated leads to severe ketoacidosis; it is less marked in the adult frequently obese type of diabetic without a tendency to ketoacidosis. These pathologic findings are in good agreement with the estimates of pancreatic insulin extractable at autopsy which will be referred to below [67].

In the opinion of the authors it would seem quite well established that in human diabetes as in experimental diabetes there exists some degree of anomaly of the insulin producing beta cells of the islets of Langerhans in a majority of instances. This statement however in no way precludes the possible frequent participation of additional etiologic factors nor does it necessarily imply acceptance of the primary nature of the pancreatic defect. Although many interesting observations have been made with regard to the possible etiologic participation of other endocrine glands in diabetes mellitus and although their participation in the clinical manifestations of the disorder is unquestioned, this topic will not be discussed here since it raises too many purely hypothetical questions and does not lend itself to reasonably succinct discussion from the major point of view of this volume i.e. from the point of view of the nature of the inherited defect. That a diabetic syndrome is frequently associated with overt dysfunctions of pituitary and/or adrenal glands is generally accepted.

## INSULIN

### *Chemistry*

The detailed analysis of the structure of insulin undoubtedly one of the major accomplishments of modern analytic protein chemistry [68] has led to the formula shown in Fig. 3.2. Insulin is a relatively small protein consisting of two polypeptide chains the shorter A chain and the longer B chain. The A chain is made up of 21 amino acid residues and the B chain of 30 residues. The two chains are linked by two disulfide bridges and there exists in addition a third internal disulfide bridge within the

and all of them result not only in increased molecular weight but also in markedly altered solubility characteristics. In the presence of zinc ions previously soluble insulin becomes insoluble and insulin precipitates in crystalline form. Heating of relatively concentrated insulin solutions at low pH results in the formation of insulin fibrils and spherites, a phenomenon quite characteristic for insulin. Some of these relationships are illustrated in Fig. 3.3, redrawn from Sanger's publication [69-70]; each hollow sphere represents a double insulin molecule of molecular weight 12,000.

The shape of the insulin molecule in aqueous solution appears to be roughly spherical. Whether this is true in complex solutions such as plasma is unknown. The detailed nature of the spatial arrangement of the polypeptide chains of insulin is still the subject of controversy. This spatial arrangement may not be intimately related to the biologic activity of insulin, since it has been reported that irreversible stretching of the chains does not grossly modify the biologic activity of the hormone [71].

### *Biosynthesis*

As yet nothing is known about the biochemical mechanisms leading to the synthesis of insulin by the beta cells of the islets of Langerhans. Accordingly, nothing is known about the presence or absence of abnormalities or deficiencies of these mechanisms in diabetes.

### *Storage*

The quantities of insulin which may be extracted from the pancreas vary from species to species [72]; in normal man the order of magnitude is 4 units per gm of pancreas [67-73]. Accordingly, it may be assumed that upwards of 200 units of insulin (i.e. about 10 mg of the pure hormone) is not infrequently stored in the beta cells of the islets of Langerhans of any given individual. This amount of insulin is potentially dangerous, and it would seem evident that it must be stored in a form precluding sudden release. As already discussed, the insulin molecule *per se* is freely soluble in water at the pH presumed to exist within cells, which is quite alkaline with relation to the isoelectric point of the hormone. It is likely, therefore, that a major part of the insulin contained in beta cells is present in a modified state resulting in its decreased solubility. Working with the giant islets of the flounder, Maske [74] has indeed shown this to be the case, since after homogenization of the islet cells, insulin activity was persistently associated with centrifugable fractions and could not be eluted therefrom.

It is of great interest that two of the mechanisms known to decrease the solubility of pure insulin (depicted in Fig. 3.3) could easily be operative in the intact beta cells of the islets of Langerhans. First, it has been

A chain—that portion of the A chain which lies between the cystine residues forming the disulfide bridges—is of special interest since it appears to be the main (possibly the only) site of molecular differentiation between species. As also indicated in the figure, insulins obtained from cattle, pig, sheep, horse and whale differ from one another only by the nature and the arrangements of the three amino acid residues contained within this disulfide ring. Pig and whale insulin are identical. Thus although this part of the molecule clearly is not essential for the biologic activity of the hormone, it is likely to be involved in its immunologic behavior<sup>1</sup>.

The unit described in Fig. 3-2 is the smallest known carrier of biologic insulin activity. Its molecular weight lies somewhat below 6,000 and its

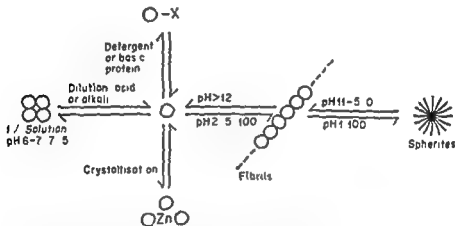


Fig. 3-3 Molecular associations of insulin. Each hollow sphere represents an insulin dimer of molecular weight 12,000. (Redrawn from Sanger [6,7].)

isoelectric point between pH 5.3 and 5.4. Where insulin is almost insoluble in water at this pH (1:100,000), this solubility increases rapidly if the pH is shifted even slightly upward or downward. In solution, insulin molecules undergo varying degrees of aggregation, and accordingly, the true molecular weight observed under a given set of conditions varies by units of 6,000. Under most conditions it would appear that at least two molecules of insulin aggregate and that further aggregation occurs by units of molecular weight 12,000. Of particular interest is the tendency of insulin to react with divalent cations, particularly zinc ions, with basic proteins such as protamines or histones, or with itself under certain conditions. These reactions are reversible.

<sup>1</sup> Recent work from Sanger's laboratory (D. M. Nichol and L. F. Smith, personal communication) indicates a further area of the insulin molecule which appears to vary between species. Human insulin is identical with pig insulin except for the C-terminal amino acid residue of the B chain, aspartic acid having replaced alanine. In rabbit insulin, the C-terminal residue of the B chain is serine.

alter the histologic appearance and in some instances the extractable insulin content of the pancreatic islets [77a] the only fully established stimulus to insulin secretion is the presence of an elevated level of blood glucose [78-81] and probably of other sugars as well. Accordingly it is to be anticipated that satisfactory hypotheses for the mechanism of insulin release will include possible mechanisms relating increased availability of glucose to accelerated release of the hormone.

An interesting mechanism has been suggested by Maske [74] linking insulin release to the interaction of stored insulin with zinc. It is known that substances which easily chelate with zinc are capable of releasing insulin from the zinc insulin complex. Among these substances are many intermediary products of cell metabolism such as citrate, oxalacetate, and glutathione. Stimulation of glucose metabolism within the islet cells may result in accumulation of one of these substances thus inducing insulin solubilization and release. On the other hand release could be effected by splitting insulin from its protein carrier or carriers as has been suggested for protamine zinc insulin [82]. Enzymes capable of effecting this separation have been described in tissues and in blood.

It should also be mentioned that a most intriguing peculiarity of beta cell metabolism has been recently described. It has been demonstrated that these cells contain appreciable concentrations of glucose 6-phosphatase an enzyme previously considered limited to liver, kidney, and intestinal mucosa [83]. It is conceivable that the activity of glucose metabolism within these cells could be in part controlled by altering the rate of glucose 6-phosphate hydrolysis relative to the rate of glucose 6-phosphate metabolism by other pathways. Although not universally accepted as yet, accumulating evidence indicates that the hypoglycemic sulfonylureas bring about an accelerated release of insulin from preformed stores [84]. In this instance accumulation of citrate [85] perhaps on the basis of accelerated glucose 6-phosphate metabolism secondary to glucose 6-phosphatase inhibition [84] has been suggested as a possible mechanism.

The present state of knowledge of insulin storage and release in human diabetes mellitus will be outlined below.

### *Transport*

After insulin has been released, it is transported in the blood stream to sites of action within the body. Since proteins frequently interact, it is not surprising to find that insulin appears to undergo several types of interactions with protein components of blood. A major portion of insulin activity or of insulin labeled with  $I^{125}$  added to serum separates electrophoretically into the  $\beta$  fraction [86-87] or by selective precipitation into fractions II and III [87-89] using Cohn's nomenclature. A quite specific type of interaction has been suggested by Antoniadou [90] i.e.

established that these cells contain an unusually high concentration of zinc [74-75]. Second it is also known that they contain several small molecular basic proteins. Indeed, Lindner [76] suggested some years ago that when insulin is extracted from fresh beef pancreas with relatively mild techniques the product obtained is insulin associated with a small molecular basic protein probably a histone. Insulin so obtained has been called 'Nativinsulin' and has been employed as a long acting form of insulin therapy just prior to the introduction of protamine zinc insulin by Hagedorn. The pharmacologic problem of artificially producing an injectable insulin which would not be rapidly soluble and absorbed from the injection site was solved by selecting protamine zinc insulin which combined the ability of both zinc and protamine (a small molecular basic protein) to produce the desired change in the solubility of the hormone.

Histologically, the beta cells of the islets of Langerhans are characterized by granules with rather specific staining properties [8]. The insulin content of any given pancreas has been shown in several studies to correlate rather well with its content in beta granules [77] as well as with its islet mass [77a]. These granules are almost certainly not insulin *per se* but they may be insulin binding or insulin storing structures. Since zinc also is present in the beta cells at least in part in granular form the possibility that the beta granules represent zinc protein cores has been raised. When relatively pure giant flounder islets were fractionated by Maske [74] the major part of zinc and insulin appeared to be associated with a mitochondrial fraction as demonstrated histologically and also by their further association with succinic dehydrogenase, a mitochondrial enzyme.

Thus in the beta cells of the islets of Langerhans a complex mechanism has been set up which primarily enables these cells to store appreciable amounts of insulin. This mechanism appears to involve the reaction of insulin with zinc and with a basic protein and may be closely associated with the site of synthesis of the hormone. It has been suggested that the appearance of the Golgi apparatus may represent a further morphologic index of the activity of insulin secretion [77b].

### *Secretion and Release*

Increased secretion of insulin could be the result either of increased synthesis with immediate release of the product or of increased release of preformed, stored insulin. In view of the evidence indicating the presence of significant amounts of stored insulin as well as variation in this amount under different conditions it seems probable that insulin secretion is controlled by mechanisms regulating its release from intracellular stores. This release is likely to involve solubilization. Although many factors (including hormonal, nutritional and neural ones) are known to

*Insulin in Human Diabetes Mellitus*

The discussion on diabetes mellitus presented in this chapter is built on the assumption that the major endocrine defect in diabetes is absolute or relative insulin deficiency. The preceding section on insulin has emphasized the complex nature of the insulin system a complexity already apparent from those components of the system about which some knowledge has accumulated and undoubtedly compounded by the components about which essentially no accepted and analyzable knowledge exists. It thus becomes evident that insulin deficiency need not be based upon a subnormal amount or number of pancreatic islets or even of insulin producing beta cells. Deficiency could equally well result from cells normal in size but abnormal in their ability to synthesize insulin or inadequately endowed with noninsulin constituents required for insulin storage or insufficiently effective in releasing insulin from its storage form when required. Lacy and Hartroft have recently emphasized the possible importance of the several membranous structures separating the beta granules from the blood stream [104a]. Deficiency could also result from an abnormal system for insulin transport from the presence of excess insulin binding or insulin neutralizing proteins from inappropriate insulin destruction or from excessive requirements for insulin secondary to for example the abnormal secretion of other hormones such as somatotropin or glucagon. Finally deficiency could follow the existence of inadequate interaction between insulin and insulin responsive tissues whether because of abnormal binding sites for insulin or because of other dysfunctions at the tissue level.

As stated above under Endocrine Defects in Diabetes, some decrease in total mass of the beta cells of the islets of Langerhans can be demonstrated with appropriate techniques in the majority of patients with diabetes mellitus [8, 60, 66]. Nonetheless in some instances this decrease is not apparent and in many instances its degree is difficult to reconcile with the requirement for removal of 90 per cent of the pancreas in the surgical induction of experimental diabetes. Accordingly great interest attaches to the careful studies of Wrenshall and his collaborators [67, 77, 105-107] who have quantitated the amount of insulin which can be extracted from the pancreas of diabetic patients at autopsy. These authors have applied a standard extraction and assay procedure to the pancreas obtained at autopsy from 170 nondiabetic and 170 diabetic patients the age variation being extensive in both groups [106, 107]. Some decrease in extractable insulin was found in the majority of diabetic patients when compared with the control group but not in all. Furthermore there appeared to be a rather clear differentiation of the total diabetic group into two subgroups. As stated by Wrenshall, the measurements clearly indicated that a gross lack of endogenous insulin is characteristic



an interaction with a small molecular basic protein. This type of interaction would thus be comparable to that existing in the beta cells and could produce a specific carrier system in equilibrium with pancreatic insulin on the one hand and tissue receptors on the other. Individuals who have been treated with insulin of animal origin for prolonged periods of time develop serum protein fractions which "bind" insulin. These appear to be  $\gamma$  globulins and thus may well represent antibodies [91-93]. Berson has reported the interesting fact that the insulin antibody complex is dissociable at least in part. There can be little doubt but that a number of proteins which occur in serum have been shown to "bind" insulin and could affect over all insulin balance either by neutralizing insulin activity or by acting as insulin "carriers".

In addition to the insulin protein interactions just described factors which affect the biologic activity of insulin without demonstrably altering its physical properties have also been reported [94-96]. The presence of some of these factors the nature of which is as yet but poorly understood has been related to diabetic ketoacidosis [96] or to the functional activity of the anterior pituitary or adrenal [97-98] glands. One of these factors appears to be lipoprotein in nature [97].

### *Inactivation*

A metabolic regulator is unlikely to be effective unless it is not only rapidly made available but also rapidly destroyed. Many tissues among which the liver and the renal tubules are outstanding are capable of inactivating insulin. Proteolytic enzymes which exhibit at least some specificity toward insulin have been described and extensive investigations of these systems as well as of factors favoring or antagonizing insulin destruction have been carried out [99-102]. It is interesting that insulin 'antibodies' of the type described above although presumably interfering with the effectiveness of insulin by preventing its interaction with insulin responsive tissues simultaneously decrease the rate of insulin destruction [91, 92].

### *Interaction with Responsive Tissues*

Although present concepts of the action of insulin will be extensively discussed in the next section it should be stated here that a considerable body of evidence indicates that at least under certain conditions insulin binding precedes or accompanies insulin effectiveness in responsive tissues [103-104]. Thus it is conceivable that insulin release, transport, action, and inactivation all are part of a system of equilibria between insulin on the one hand and insulin receptors on the other. Evidence presently accumulating in this laboratory in collaboration with H. N. Antoniadou suggests that indeed this might be the case.

been clearly outlined by Field [114]. It should perhaps be recalled here that as yet no convincing evidence for genetic separation of these two clinical types has been presented.

That nonresponsiveness of some or all insulin dependent tissues may be involved in some instances of diabetes mellitus has been recently suggested in a number of instances particularly in the rare syndrome of *associated lipomatrophy and diabetes* first described by Lawrence [115-119]. Furthermore the recent report by Miller [117] of the occurrence of lipotrophic diabetes in two siblings suggests the possibility that this syndrome is genetically distinct.

## METABOLIC EFFECTS OF INSULIN

Soon after Banting and Best [120] reported in 1921 the preparation of pancreatic extracts which were successful in lowering blood glucose and in prolonging the life of pancreatectomized dogs insulin became available to several laboratories in Canada, the United States and Great Britain. A complete bibliography [121] published in 1923 contained only 15 articles but from these it was already evident that insulin *primarily* caused an increased rate of extraction of glucose from blood. Hepburn and Litchford [122] soon demonstrated an effect *in vitro* namely a three to fourfold increase in glucose uptake by the isolated perfused heart after insulin was added to the system. It is not surprising that this preparation should have been among the first used for prior to the availability of insulin it had been reported that hearts excised from diabetic animals extracted less glucose from the perfusing medium than did normal hearts [123] and several years before the achievement of Banting and Best Clark [124] reported that the connection of a perfused pancreas into the perfusing circuit accelerated the rate of glucose disappearance<sup>1</sup>.

By 1925 it had been demonstrated that insulin accelerates the rate of glucose uptake by extrahepatic tissues other than the heart as measured by arteriovenous differences across an extremity [125] or by an increased rate of disappearance in the hepatectomized dog [126] and in the eviscerated cat [127]. It had also been shown that insulin acutely increases the respiratory quotient and Kellaway and Hughes [128] suggested that much of the disappearing glucose was converted to an oxygen poor substance such as fat.

Since the pioneering studies mentioned above the bibliography of insulin has become diversified and extraordinarily extensive yet it would still seem that insulin primarily effects an accelerated glucose uptake by certain tissues. With the refinement of physiologic techniques and especially since the advent and now wide spread use of radioactive isotopes numerous metabolic processes have been reported to be altered following the addition of insulin to experimental systems whether to

of human beings who acquire diabetes during the period of normal growth (approximately the first twenty years of life). Diabetes diagnosed thereafter differed in that a considerable amount of insulin was present in the pancreas at autopsy in a majority of such persons [106]. Mean values presented in 1952 are shown in Fig. 3-4.

Pancreatic insulin extractable at autopsy, need not of course reflect the secretory ability of the pancreatic islets. Indeed this measurement is more likely to relate to the adequacy of insulin storage. It is of interest therefore to find that several investigators have reported the presence

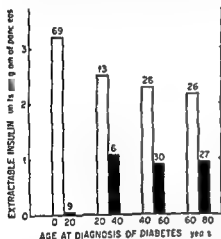


Fig. 3-4 Variation in the average extractable insulin of human pancreas at autopsy in diabetes (black strips) with age at diagnosis of diabetes. Values for normal nondiabetic controls at death are shown as white strips. The number of subjects in each group is shown above the strip. (From Wrenshall [67].)

of subnormal quantities of circulating insulin like activity in serum or plasma of patients whose diabetes developed during the period of normal growth while circulating insulin like activity was near normal or greater than normal in patients with adult onset of the disorder [108-109]. Other observers have failed to find uniformly decreased plasma insulin like activity in juvenile diabetic patients [110-111].

On the basis of the studies just summarized as well as others [112-113] there is a widespread tendency to differentiate two major clinical types of diabetes in man. The first type declares itself most frequently during the period of growth and is typically associated with weight loss, severe ketoacidosis on insulin withdrawal, normal sensitivity to

insulin or even hypersensitivity, nonresponsiveness to hypoglycemic sulfonylureas, and evidence of decreased pancreatic and plasma insulin content. The second type declares itself preferentially during adult life and is typically associated with obesity, absence of severe ketoacidosis on insulin withdrawal, subnormal sensitivity to insulin, responsiveness to hypoglycemic sulfonylureas, and poor if any evidence of decreased pancreatic and plasma insulin content. Intermediate forms exist and indeed are frequent. It is entirely conceivable that these two clinical types of diabetes differ primarily from a quantitative point of view and represent complete (growth-onset ketotic) or incomplete (adult nonketotic) insulin deficiency. However, it is equally conceivable that the adult nonketotic type of diabetes in man represents relative insulin deficiency secondary to the existence of other etiologic factors resulting in an increased requirement for insulin. The present status of this important controversy has recently

Emden [132] and Meyerhof [133] 20 years later defined the part played by phosphorylated intermediates in the process of fermentation. Since then it has become apparent that mammalian organisms likewise require the formation of phosphate esters prior to the stepwise metabolism of carbohydrates.

Before glucose can be metabolized it has to enter the cell. Once inside the cell it is phosphorylated. The enzyme which catalyzes the phosphorylation of glucose is hexokinase and the reaction as carried out in tissue extracts or purified enzyme systems requires high-energy phosphate (an acid anhydride) to form glucose 6-phosphate (Fig 3-5). It also is favored by the presence of magnesium and potassium ions. The enzyme has been found in all tissues examined. In liver it is frequently termed less generally 'glucokinase' since this tissue also contains

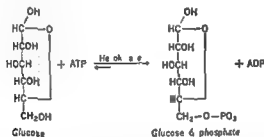


Fig 3-5 Activation of glucose to glucose 6-phosphate

specific kinases for maltose and fructose. The reaction is exergonic and thus predominantly unidirectional (at physiologic concentrations of all reactants) toward the formation of glucose 6-phosphate.

### *Metabolism of Glucose 6 Phosphate*

Glucose 6-phosphate has at least five different metabolic pathways open to it. Some of these pathways are common to all tissues while some tissues lack the enzymes required for one or more of them. Each tissue characteristically appears to exhibit its own unique pattern which may vary depending upon the organism's hormonal or nutritional state at any given time. Schematically these pathways are shown in Fig 3-6.

### *Glucose 6 Phosphatase*

The first and simplest route open to glucose 6-phosphate metabolism is that catalyzed by the single enzyme glucose 6-phosphatase resulting in the cleavage of glucose 6-phosphate into free glucose and inorganic phosphate. This reaction is the principal source of nonalimentary glucose in the mammalian organism and provides circulating glucose during periods of carbohydrate deprivation. The enzyme is present primarily in liver although its concentration is similar in the kidney but because

the total animal an eviscerated or hepatectomized preparation a perfused organ or limb (either isolated or *in situ*) an isolated tissue preparation *in vitro* or even in some instances separated cells subcellular particles or enzymes. The number and diversity of the reported effects suggest that insulin controls a single widespread and fundamental reaction which in turn affects numerous subsidiary reactions or that it exerts direct effects upon multiple physiologic functions. To date experimental evidence favors the former concept namely that insulin primarily affects a single process carbohydrate entry into cells.

### *Primary and Secondary Effects*

Current developments in biochemistry and endocrinology have tended to support the thesis that hormones exert their primary action upon a single enzymatic reaction or reaction type and that subsequent effects are secondarily linked to this primary event. An excellent specific example is that of the increased hepatic phosphorylase activity demonstrated after epinephrine or glucagon administration [129]. In this instance the primary effect of epinephrine and glucagon is that of inducing the formation of 3',5'-cyclic adenosine nucleotide a substance which favors the accumulation of the active form of the activating enzyme (dephosphophosphorylase kinase) which in turn catalyzes the phosphorylation of inactive phosphorylase (dephosphophosphorylase) to active phosphorylase [130]. The increased phosphorylase then accelerates the catalysis of glycogen breakdown and in the presence of glucose 6-phosphatase raises blood glucose. In this instance therefore it is possible to trace in some detail the links which connect the observed physiologic effect of epinephrine and glucagon i.e. hyperglycemia with the primary or molecular site of action of these hormones.

It is tempting to speculate that insulin similarly is primarily concerned with a single important and obviously vital reaction and that the numerous observed metabolic effects are merely reflections of this reaction.

In order to discuss primary and secondary metabolic alterations produced by insulin (or by insulin lack) some pathways of carbohydrate fat and protein metabolism and the intimate interrelations between these pathways must first be sketched. The following brief section will stress the interdependence of the pathways discussed rather than the individual enzymatic reactions themselves. For further discussion the reader is referred to the bibliography and to standard biochemical references.

## SOME PATHWAYS OF INTERMEDIARY METABOLISM

### *Phosphorylation of Glucose*

Harden and Young [131] at the turn of the century described the role of phosphate in the utilization of sugar by living organisms and

leading to glycogen breakdown [139] Since enzymes only accelerate the attainment of a given equilibrium this observation similarly suggested that glycogen synthesis must occur by a different route while phosphorylase under physiologic conditions serves to catalyze glycogen breakdown only [140]

Beloff Chain [141] interpreted a series of experiments in muscle and liver as suggesting that glycogen synthesis occurs by direct polymerization of glucose to disaccharides trisaccharides and progressively larger molecules rather than via phosphorylated intermediates In 1957 Leloir and Cardini [142] demonstrated glycogen synthesis in liver preparations in the presence of uridine diphosphoglucose as cofactor and demonstrated in a subsequent publication that this pathway could account for the rate of glycogen synthesis prevailing in liver in vivo The uridine diphosphoglucose (UDPG) pathway has also been demonstrated in muscle and its equilibrium has been reported to favor glycogen synthesis [138] It is as yet too early to evaluate the detailed importance of the UDPG-catalyzed mechanism in the intact animal or in tissues other than liver and muscle The pathways of glycogen synthesis and breakdown which have been discussed are outlined in Fig 3 7

Once glycogen has been formed the 1 4 glycosidic links may be altered by branching enzyme (amylol-1 4 1 6 transglycosidase) to form 1 6 links thereby originating new chains of 1 4 links Thus glycogen consists of a central reducing aldehyde from which spring chains of 1 4 links and less frequent 1 6 links or branch sites The ratio of 1 4 links to 1 6 links is approximately 8 1 but this figure is an average and there is probably considerable inhomogeneity [143] As expected the outer tiers of the glycogen molecule are metabolically more active as has been shown by the isotopic carbon studies of Stetten and Stetten [144]

In the breakdown (or phosphoryolysis) of glycogen by phosphorylase successive 1 4 links are cleaved to form glucose 1 phosphate until a 1 6 link is encountered To rupture this bond another enzyme amylol-1 6 glucosidase is required This results in the formation not of glucose 1 phosphate but of free glucose This free glucose is presumably rephosphorylated in peripheral tissues in liver it may be a source of blood glucose though obviously a much less important one than that resulting from glucose 6 phosphate activity Much additional information concerning glycogen metabolism is presented in Chap 6 Glycogen Deposition Diseases

### *Glycolysis*

The third route of glucose 6-phosphate metabolism is the now classical glycolytic route (Fig 3 8) also termed the *Embden Meyerhof* or *Embden Meyerhof Larnas Cori* pathway after the investigators who contributed to its elucidation Glucose 6-phosphate is isomerized to

of its smaller size the kidney contributes no more than one tenth of the production of blood glucose. The enzyme has also been described in intestinal mucosa and placenta and to a much lesser extent in other tissues [134]. Its probable presence in the beta cells of the islets of Langerhans has already been discussed. Curiously, the enzyme is not found in the soluble fraction as are all the other enzymes directly involved in the metabolism of glucose 6-phosphate but is associated with the microsomes [135]. The physiologic role of this enzyme in controlling the level of blood glucose will be discussed below, in the section devoted to liver metabolism.

### *Synthesis of Glycogen*

A second pathway open to glucose 6-phosphate in glycogen synthesis is Glucose 6-phosphate first forms Glucose 1-phosphate, in the presence of

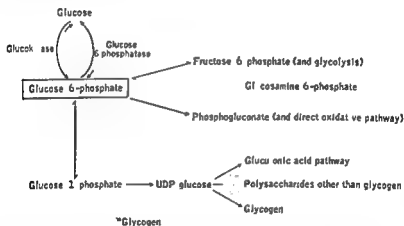


Fig. 3-6 Metabolic pathway of glucose 6-phosphate

phosphoglucomutase a reaction in which glucose 1,6-diphosphate is a necessary cofactor. In the presence of active phosphorylase, glucose 1-phosphate condenses with the nonreducing end of a glycogen chain forming a new 1,4-link with loss of inorganic phosphate. Until recently it was generally considered that this reaction governed both glycogen synthesis and mobilization, the direction of the net reaction depending primarily on the relative concentrations of glucose 1-phosphate and inorganic phosphate [136]. When the intracellular concentrations of these two substances were actually measured, it was found that their relative concentrations were such as always to favor glycogenolysis [137-138]. Furthermore, it was well known that epinephrine-induced or glucagon-induced increases in phosphorylase activity always increased glycogenolysis, never the synthesis of glycogen. This indicated that phosphorylase activity is rate limiting only in the sequence of events

fructose 6-phosphate which is further phosphorylated to form fructose 1,6-diphosphate in the presence of 6-phosphofructokinase. This phosphorylation similar to the initial phosphorylation of glucose requires the donation of high-energy phosphate from ATP to form a low-energy ester. The reaction is essentially unidirectional. The reverse reaction, the conversion of fructose 1,6-diphosphate to fructose 6-phosphate and inorganic phosphate, is catalyzed by another enzyme, fructose 1,6-diphosphatase, also an essentially unidirectional reaction. The participation of two enzymes in the control of the interconversion of fructose 6-phosphate and fructose 1,6-diphosphate is of considerable importance.

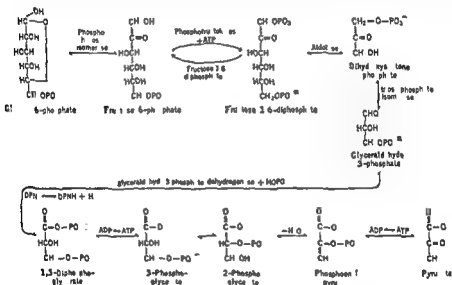


Fig 3.8 Pathway of glycolysis

since it provides a means of controlling the overall rate and direction of the glycolytic pathway. Tissues which are mainly glycolytic such as muscle or brain, contain the highest concentration of phosphofructokinase [145]. Conversely, high levels of fructose 1,6-diphosphatase would be expected in whatever tissue exhibits the greatest degree of gluconeogenesis from three-carbon precursors. The best source of this enzyme is indeed the liver [146].

Fructose 1,6-diphosphate is then split in the presence of aldolase into two phosphorylated three-carbon intermediates, the upper three carbons forming dihydroxyacetone phosphate and the lower three, glyceraldehyde 3-phosphate. The two compounds are interconvertible in the presence of the enzyme triose phosphate isomerase. Glyceraldehyde 3-phosphate is then dehydrogenated in the presence of phosphoglyceraldehyde de-



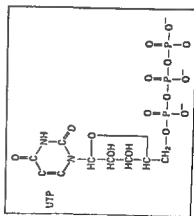
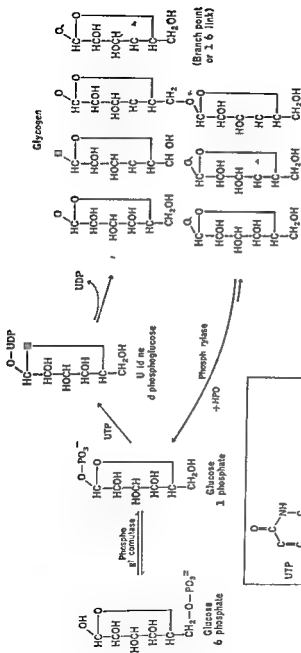


Fig 3.7 Pathway of glycogen synthesis and breakdown



hydrogenase and diphosphopyridine nucleotide (DPN) as hydrogen acceptor with the simultaneous introduction of a second phosphate into the molecule to form 1,3-diphosphoglycerate. This compound subsequently loses one phosphate residue in the presence of ADP to form 3-phosphoglycerate and ATP. In the succeeding steps the remaining phosphate is transferred to the 2 position and 2-phosphoglycerate is transformed into phosphoenolpyruvate with the loss of 1 molecule of water. In the final step phosphoenolpyruvate with ADP, is converted to pyruvate and ATP.

In summary the conversion of 1 mole of glucose to 2 moles of pyruvate requires 1 mole of ATP for each of the phosphorylations, while the metabolism of each of the two trioses to pyruvate yields 2 moles of ATP and 1 mole of reduced DPN. In the absence of oxygen the hydrogens of reduced DPN can be conveniently disposed of by transferring them to pyruvate in the presence of the enzyme lactic acid dehydrogenase resulting in the formation of lactate and in the regeneration of oxidized DPN. The over all reaction under these conditions may be written



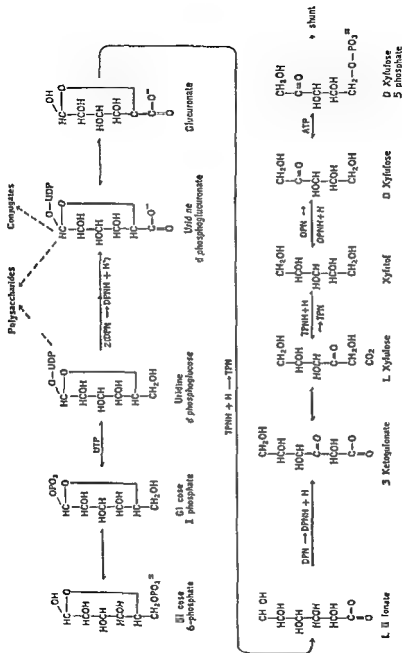
For each mole of glucose metabolized by the glycolytic pathway there is a net production of 2 moles of ATP. This energy accounts for a small fraction only of the total energy available from glucose but it is available in the absence of oxygen. Therefore the term most commonly used to refer to this sequence of reactions is *anaerobic glycolysis* since it can indeed proceed "anaerobically".

#### *Phosphogluconate oxidative Pathway*

Names used for this route of glucose 6-phosphate metabolism include in addition to *phosphogluconate oxidative pathway* the terms *direct oxidative pathway*, *phosphogluconate pathway*, *Lipmann-Dickens pathway*, *pentose phosphate pathway*. The colloquial term frequently applied to this metabolic route is that of the "shunt".

Glucose 6-phosphate first undergoes dehydrogenation to form 6-phosphogluconolactone, a cyclic ester which in turn is enzymatically hydrolyzed to 6-phosphogluconate. Next 6-phosphogluconate is dehydrogenated and decarboxylated producing ribulose 5-phosphate. These two initial reactions of the pathway result in the removal of the aldehyde carbon of glucose as  $\text{CO}_2$  and in the further removal of four hydrogens. The specific acceptor for these hydrogens is triphosphopyridine nucleotide (TPN) resulting in the formation of two molecules of reduced TPN. The importance of this by-product of the oxidative steps of this pathway will be shown during the discussion of the synthesis of fatty acids.

The further metabolism of ribulose 5-phosphate as shown in Fig. 9 requires the rearrangement of two molecules, one isomerized into ribose 5-phosphate and the other epimerized into xylulose 5-phosphate. The



**Fig. 10** Glucuronic acid pathway

and glyceraldehyde 3 phosphate. The over all reaction may be written



The initial oxidative steps are essentially irreversible hence the metabolism of glucose 6-phosphate by this pathway proceeds only in the direction of pentose phosphate formation. However, all the other steps are reversible. For example by the trans aldolase reaction fructose 6-phosphate can transfer its upper three carbons to erythrose 4 phosphate to form sedoheptulose 7 phosphate or by the transketolase reaction it can transfer its upper two carbons to glyceraldehyde 3 phosphate to form xylulose 5-phosphate and erythrose 4 phosphate. In tissues such as muscle which lack significant amounts of the two enzymes needed to dehydrogenate glucose 6-phosphate ribose synthesis occurs by transketolation and transaldolation from fructose 6-phosphate.

### *Glucuronic Acid Pathway*

At the time of this writing, it is difficult to assess the quantitative importance of the glucuronic acid pathway in the total organism or in individual tissues. The existence of this pathway has been established beyond reasonable doubt by Horecker and Hiatt [147]. It first requires the conversion of glucose 6-phosphate to glucose 1 phosphate which subsequently reacts with uridine triphosphate (UTP) to form uridine diphosphoglucose (UDPG) and pyrophosphate. Uridine diphosphoglucose may undergo several reactions including the formation of glycogen, epimerization at the fourth carbon of glucose to form uridine diphosphogalactose, possibly epimerization and metabolism in other positions to form sugars such as idose, mannose or galactosamine and in the glucuronic acid pathway oxidation at the sixth carbon of glucose with two molecules of DPN as coenzyme to form uridine diphosphoglucuronic acid (UPDGA) and two molecules of DPNH. This UDPGA may then be used in the synthesis of polysaccharides and mucopolysaccharides or it may serve as the glucuronic acid donor in conjugating reactions with substances such as bilirubin, phenols or steroids or it may be further metabolized as shown in Fig. 3-10. In this sequence of events uridine diphosphate is split off and free glucuronic acid is reduced in the first position to form L-gulonic acid. This in turn is oxidized to 3 ketogulonic acid or 2 ketogulonic acid then decarboxylated to L-xylulose. In animals such as the rat 3 ketogulonic acid or its isomer 2 ketogulonic acid appears to be the precursor of ascorbic acid. L-xylulose is reduced to xylitol, a symmetric molecule, then reoxidized to form D-xylulose. After phosphorylation in the presence of D-xylulose kinase and ATP, xylulose 5 phosphate can be metabolized by the reactions described in Fig. 3-9, i.e. the reactions of the phosphogluconate-oxidative pathway.

lenged since it has been shown that the presence of  $\text{CO}_2$  is a necessary requirement the intermediate product formed being malonyl CoA [148] Malonyl CoA then condenses with acetyl CoA (or more generally with an acyl CoA) to form the corresponding branched chain  $\alpha$  carboxyl  $\beta$  keto fatty acid with subsequent reduction of the keto group dehydration and reduction of the double bond and finally cleavage to  $\text{CO}_2$  of the same carboxyl originally derived from  $\text{CO}_2$ . The over all reaction

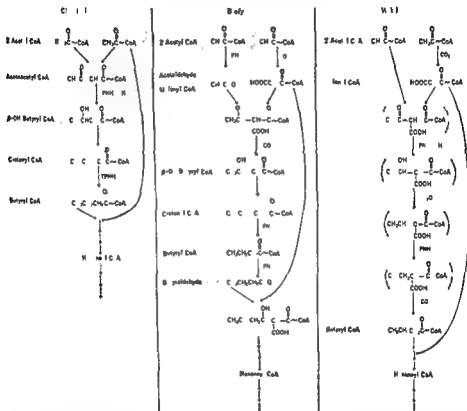


Fig. 1-11 Pathways of fatty acid synthesis (early 1960)

thus also results in elongation of the acyl residue by two carbons without evidence of the  $\text{CO}_2$  carbon in the end product! In the isolated system *TI* *VI* again series as corn *yme* in the *re* *luc* *te* *p*s. Although clarification of the quantitative importance of this pathway is still required it is of interest to consider that if fatty acids were synthesized primarily by the malonyl pathway and primarily catabolized by reversal of the classical pathway synthesis and breakdown could be more readily controlled the over all pattern being analogous to synthesis of glycogen

### *Metabolism of Pyruvate*

Under aerobic conditions the pathways of glucose dissimilation converge to form pyruvate. Pyruvate is oxidatively decarboxylated to form acetyl coenzyme A (acetyl CoA) and DPNH. This essentially irreversible and complex step requires the presence of a conjugate of thiamine and lipoic acid and of several fractions with enzymatic activity. Acetyl CoA has several pathways open to it of which four are of major quantitative importance: oxidation in the tricarboxylic acid cycle; incorporation into long chain fatty acids; incorporation into cholesterol and metabolism to acetoacetate and  $\beta$  hydroxybutyrate.

### *Tricarboxylic Acid Cycle*

Acetyl CoA condenses with oxaloacetate to form citric acid which is then metabolized further in the presence of the enzymes which comprise the tricarboxylic acid (TCA) cycle also called the Krebs or the citric acid cycle. Each turn of this cycle leads to the incorporation of one molecule of acetate while two molecules of  $\text{CO}_2$  are given off and while two molecules of DPNH, one molecule of TPNH and one high energy phosphate bond are generated and two further hydrogen atoms are directly incorporated into the respiratory enzyme chain. More than 90 per cent of the energy of the glucose molecule is derived by the further metabolism of pyruvate by the TCA cycle.

### *Synthesis of Fatty Acids*

Acetyl CoA may condense with a second molecule of acetyl CoA cleaving off free coenzyme A with formation of acetoacetyl CoA. In fatty acid-synthesizing tissues (Fig. 3.11) and according to concepts generally accepted until very recently, acetoacetyl CoA is then reduced stepwise first in the presence of DPNH to form  $\beta$  hydroxybutyryl CoA which is dehydrated to crotonyl CoA. Crotonyl CoA is then reduced in the presence of TPNH to butyryl CoA. The process is then repeated two carbons at a time beginning by condensation with another molecule of acetyl CoA to form the CoA derivative of the  $\beta$  keto acid with two more carbons with subsequent reduction to the  $\beta$  hydroxy acid, dehydration to the unsaturated acid and finally reduction to the saturated fatty acid. After a predetermined chain length has resulted from these successive elongations free coenzyme A may be regenerated while esterification of the fatty acid residues with 1- $\alpha$  glycerophosphate may lead to the formation of phosphatidic acids and subsequently to the formation of triglycerides or phospholipids. Esterification of the fatty acid residues may also occur with other alcohols such as cholesterol.

Recently this classical pattern of fatty acid synthesis has been chal-

mevalonic acid; once this intermediate is formed cholesterol synthesis appears to proceed almost spontaneously [150]. The problem of cholesterol synthesis and metabolism is more fully discussed in Chaps. 16 and 20.

### Ketogenesis

Acetoacetyl CoA and  $\beta$  hydroxybutyryl CoA instead of undergoing further metabolism to fatty acids or cholesterol by successive elongation of their carbon chain may also form free acetoacetate and  $\beta$  hydroxybutyrate. Liver and kidney are equipped with a series of enzymes effecting 'deacylation' and it has recently been suggested by Lynen [151] that the reaction might be a complex one involving first the synthesis of  $\beta$  hydroxy  $\beta$  methyl glutaryl CoA with subsequent liberation of acetoacetate, acetyl CoA reentering the acetyl CoA pool. This reaction sequence resulting in the accumulation of acetoacetate becomes quantitatively important when acetoacetyl CoA accumulates under conditions which do not favor the synthesis of fatty acids and to a lesser extent cholesterol. This is the case for instance in fasting or in the diabetic state as will be further emphasized below. When acetoacetate accumulates in acetoacetate-forming tissues it diffuses into the blood stream and is carried to all tissues. Before being utilized for further oxidation or for synthetic reactions it has to be reactivated either (in all tissues) by direct reaction with coenzyme A in the presence of ATP or (primarily in muscle) by transfer of coenzyme A from succinyl CoA formed in the tricarboxylic acid cycle. The latter reaction is energetically more economical and whereas it does not occur in liver it predominates in muscle thereby establishing a transport system for acetoacetate from liver to muscle under appropriate conditions.

### Dicarboxylic Acid Shuttle

Another important metabolic link between glycolysis and the TCA cycle is frequently termed the *dicarboxylic acid shuttle*. Although the feasibility of the direct reaction has been recently suggested [152] the direct conversion of pyruvate to phosphoenolpyruvate is difficult since the equilibrium greatly favors the formation of pyruvate because of the free-energy levels of the reactants [152]. Studies with carbon 14-labeled substrates have shown that tissues which synthesize glucose (e.g. liver, placenta) by-pass this energy block by a reductive carboxylation of pyruvate to malate in the presence of TPNH. Malate, an intermediate of the TCA cycle, is oxidized by  $\text{DIN}$  to oxalacetate which is in turn decarboxylated and phosphorylated in the presence of inorganic guanosine triphosphate to form phosphoenolpyruvate. Thus the direct conversion of pyruvate to phosphoenolpyruvate is bypassed and in the process TPNH is oxidized to  $\text{TPN}$  and  $\text{DIN}$  reduced to  $\text{DINH}$  as shown in Fig. 3-13.



by the uridine diphosphoglucose pathway and breakdown by the phosphorylase mechanism

### Synthesis of Cholesterol

Acetyl CoA is also used for the synthesis of cholesterol. Through a series of reactions, as yet incompletely understood [19] acetyl CoA condenses with acetoacetyl CoA to form  $\beta$ -hydroxy  $\beta$ -methyl glutaryl CoA which is reduced by TPNH to mevalonic acid which in turn

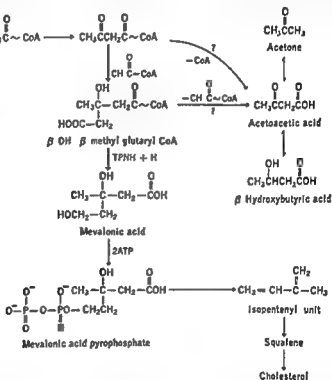


Fig 3-12 Pathways of cholesterol synthesis and ketogenesis

decarboxylated to form an 'isoprenoid' i.e. an isopentenyl derivative similar in structure to isoprene as shown in Fig 3-12. Three of these isoprenoid units condense to form a 15-carbon intermediate of which two condense end to end to form squalene which folds, condenses, splits off three methyl groups and after a single reduction in the side chain results in the formation of cholesterol [19]. Cholesterol may then undergo many further reactions which include the synthesis of steroid hormones, bile acids or vitamin D or it may remain unchanged as cholesterol free or esterified. Recent studies have suggested that the rate limiting step in this sequence of reactions is the formation of



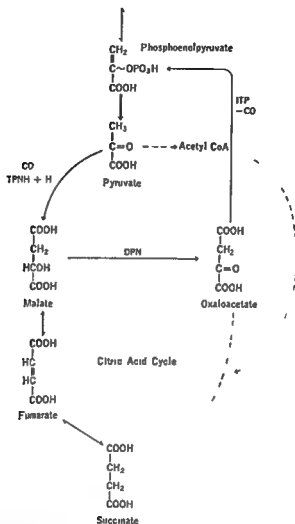


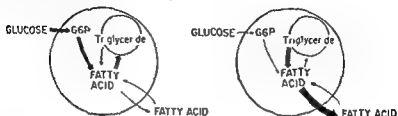
Fig. 3-13 Dicarboxylic acid shuttle

### Hydrogen Balance

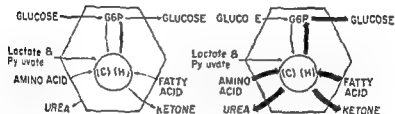
Although it is customary to think of intermediary metabolism in terms of the carbon atoms involved or in terms of oxygen used, it is becoming apparent that one of the major means used for the regulation of intermediary metabolism is the control of hydrogen flow or balance between substrates [164] intermediates and water with the help of some highly specific coenzymes among which DPN and TPN are of particular importance to this discussion. A summary of the roles of DPN and TPN in the reactions which have been outlined is shown in Fig. 3-14. It should again be stressed that the conversion of acetoacetate

must be assumed that glucose phosphorylation and dephosphorylation are occurring at identical rates under these conditions. Below this level of glycemia liver produces glucose, and above this level glucose is taken up and deposited as glycogen. Glucose 6 phosphatase activity is increased

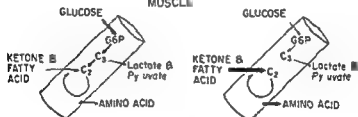
## ADIPOSE



## LIVER



## MUSCLE



NORMAL

DIABETIC

Fig 3-15 Effect of the diabetic state on some pathways of intermediary metabolism in liver, muscle and adipose tissue

by fasting and is also elevated in diabetes. The effective activity of its opposing partner, glucokinase, is measured by the phosphorylation mechanism, is decreased by fasting or in diabetes. As a result, in the diabetic state, liver may continue to produce glucose even in the presence of severe hyperglycemia, whereas the normal liver removes glucose and deposits it as glycogen under these conditions [1,7].

Likewise a tissue actively fixing  $\text{CO}_2$  as in hepatic gluconeogenesis from pyruvate would be expected to exhibit relatively poor lipogenesis and indeed lipogenesis in liver appears to be roughly inversely proportional to the activity of simultaneously occurring gluconeogenesis.<sup>1</sup>

The enzymatic interconversion of DPNH and TPNH has been described in several tissues. The enzyme involved transhydrogenase does not appear to be sufficiently active to equilibrate effectively intracellular DPNH and TPNH. Recent evidence suggests that the activity of this interconversion may play a major role in the hormonal regulation of metabolic pathways. Thus placental transhydrogenase is activated by estrogens [155] and there is evidence that other steroids may likewise exert their metabolic effects by acting as cofactors for this enzyme in specific tissues.

### METABOLISM IN INDIVIDUAL TISSUES

The previous paragraphs have briefly outlined some aspects of over all metabolic pathways, their direct interrelations and the indirect integration achieved by the coenzymes DPNH and TPNH. In the following section three typical tissues will be discussed: liver, muscle and adipose tissue. These are selected from a metabolic viewpoint as quantitatively the most important tissues for over all energy metabolism and also because they exemplify three different patterns of metabolism. Furthermore they are the tissues most actively studied in diabetes. Liver is primarily gluconeogenic, muscle is primarily glycolytic (its main function is the derivation and storage of chemical energy which can be immediately or eventually converted to mechanical energy) and adipose tissue controls the synthesis and storage of fatty acids as triglyceride and the mobilization of the fatty acids for release into the blood stream. Some of these relationships, both in the normal and in the diabetic state, have been summarized in Fig. 3.1.

#### Liver

The role of the liver in carbohydrate metabolism, as originally and correctly defined by Claude Bernard over 100 years ago, is twofold. It is able to store carbohydrate as glycogen and to release this carbohydrate into the circulation when needed. It is also able to convert noncarbohydrates such as proteins, amino acids, or intermediates such as glycerol or lactate into glucose and glycogen. The enzymatic machinery of the liver is therefore geared to these activities. As stated previously, glucose 6-phosphatase is present in liver and catalyzes the final step resulting in the production of blood glucose.

Soskin and his collaborators [156] have demonstrated that glucose uptake or production by liver from normal fed animals ceases at the blood glucose concentration of approximately 150 mg per 100 ml. Thus it

lates as acetoacetyl CoA and ultimately as acetoacetate. From this one might tentatively conclude that *ceteris paribus* the generation of one acetoacetate molecule from fatty acid catabolism could account for the synthesis of two glucose molecules from pyruvate.

### Muscle

With regard to the aspects of intermediary metabolism considered here muscle metabolism is relatively simpler than liver metabolism. Muscle appears to lack the enzymes required for the oxidative steps of the shunt although it does exhibit other enzymatic mechanisms of this pathway and probably synthesizes pentoses by means of the transaldolase and transketolase reactions [101]. In muscle there is essentially no fatty acid synthesis (a finding consonant with the absence of the TPNH generating oxidative steps of the shunt) and studies with isotopic tracers have revealed the probable absence of the dicarboxylic acid shuttle [102]. What little pyruvate is incorporated into muscle glycogen is apparently incorporated by direct reversal of the equilibrium between phosphoenolpyruvate and pyruvate. Finally muscle lacks glucose 6-phosphatase and is therefore a purely glucose utilizing never a glucose-producing tissue. Besides glucose muscle readily metabolizes ketone bodies and fatty acids and *in vitro* studies have shown that in certain metabolic states such as diabetic acidosis all the metabolic energy of muscle may be derived from the metabolism of these nonglucose fuels [103].

### Adipose Tissue

Adipose tissue represents another pattern of metabolism, one centered around fatty acid synthesis and release. Experiments with isotopically labeled carbon have shown that the major part of mammalian fat deposits is synthesized directly in adipose tissue from glucose and other precursors [104]. Glucose is a particularly effective fatty acid precursor in this tissue, half the glucose being metabolized by way of the shunt in order to generate the large amounts of required TPNH [105]. Triglycerides in the form of chylomicrons are probably to a large extent incorporated directly into adipose tissue lipid [106] although the ester bonds may well be hydrolyzed and resynthesized in the process. The second important metabolic function of adipose tissue concerns the release of fatty acids from the tissue into the circulation, primarily in the form of albumin bound nonesterified (NEFA) or unesterified (UFA) or free fatty acids. Much research is currently in progress concerning the mechanism of this release. In general it appears that adipose tissue releases fatty acids especially during episodes of carbohydrate deprivation [107]. The carbohydrate deprivation may be absolute as in the fasting state or it may be relative as in the diabetic state with hyperglycemia but

In regard to the other step in glycolysis which is controlled by two opposing enzymatic reactions it has already been stated that phosphofructokinase activity in liver is lower than in any other tissue in which it was assayed [145]. Conversely, fructose 1,6-diphosphatase is readily demonstrated in liver and its activity increases in fasting in diabetes or following glucocorticoid administration [146]. Likewise the activity of the dicarboxylic acid shuttle is great in liver and is increased in fasting and in diabetes in order to permit accelerated synthesis of phosphoenolpyruvate from pyruvate. In summary, therefore, within the sequence of reactions which comprises the glycolytic pathway, each step for which two separate enzymes or enzyme systems are provided favors gluconeogenesis in hepatic tissue and this tendency is further enhanced by fasting or in the diabetic state.

With respect to the direct oxidative pathway it has been repeatedly shown in studies with glucose specifically labeled with  $C^{14}$  that twice as much carbon 1 as carbon 6 is recovered in  $CO_2$ . This suggests that one-half the glucose is metabolized by the shunt. However, subsequent studies in which glucose carbon was recovered in fatty acids and in glycolytic intermediates in addition to  $CO_2$  suggest that the amount of glucose 6-phosphate so metabolized is more likely of the order of magnitude of 5 to 10 per cent [158]. In the liver of the diabetic animal this percentage increases somewhat but the absolute amount of glucose 6-phosphate metabolized is well below normal [159].

Whether fatty acid synthesis proceeds primarily according to classical concepts (Fig. 3-11) or by the malonyl CoA pathway,  $TPNH$  is a required hydrogen donor at one or more of the reductive steps involved. Indeed, as particularly emphasized by Siperstein [160], the availability of  $TPNH$  may well play a necessary and possibly rate-limiting role in hepatic lipogenesis. It is therefore not surprising to find that since only one-tenth the normal amount of glucose is metabolized by the  $TPNH$  generating pathway in diabetes, lipogenesis in the liver of the diabetic animal is markedly reduced. Also, since gluconeogenesis from pyruvate primarily occurs by way of the dicarboxylic acid shuttle, i.e., reactions resulting in the utilization of  $TPNH$ , accelerated gluconeogenesis from amino acids, lactate, or pyruvate is likely to be associated with less fatty acid synthesis, as indeed appears to be the case.

It should be pointed out that the role of fatty acid synthesis in liver is probably relatively minor and less important than fatty acid oxidation, which provides in this tissue for the necessary reductive environment required for the synthesis of glucose. To synthesize one molecule of glucose from two molecules of pyruvate requires four hydrogen atoms. The oxidative generation of one molecule of acetyl CoA from a long-chain fatty acid results in the availability of four hydrogen atoms in a metabolically appropriate form. If total oxidation is limited, acetyl CoA accumu-

TABLE 3 ■ RESPONSIVENESS OF INDIVIDUAL TYPES TO INULIN

| T                          | Responsiveness<br>to inulin | Major inulin effect   | Effect or lack of effect<br>detected |          |        | Concentration<br>of inulin<br>required<br>microns/ml | Time<br>required for<br>initiation<br>of effect |
|----------------------------|-----------------------------|---|--------------------------------------|----------|--------|--|---|
|                            |                             |   | In vivo                              | In vitro | In man |  |   |
| Muscle                     | Present                     | Increased glucose uptake in<br>creased glycogen is also in-<br>creased uptake of amino acid<br>increased incorporation of<br>amino acids into protein | Yes                                  | Yes      | Yes    | 10-1 000<br>10                                       | Min   |
|                            | Present                     |   | Yes                                  | Yes      | Yes    |  |   |
| Adipose tissue             | Present                     | Increased glucose uptake and<br>oxidation increased lipogenesis<br>in the presence of glucose also<br>decreased release of fatty acids                | Yes                                  | Yes      | Yes    | 10   | Min   |
|                            | Present                     |   | Yes                                  | Yes      | Yes    |  |   |
| Adipose gland<br>(tasting) | Present (rat)               | Increased lipogenesis in the pres-<br>ence of glucose   | Yes                                  | Yes      | Yes    | 9 000  | Min   |
|                            | Absent (sheep)              |   | Yes                                  | Yes      | Yes    |  |   |
| Lactating<br>mammary gland | Present                     | Increased glucose uptake  | Yes                                  | Yes      | Yes    | 10 000<br>10 000                                     | 1 hr<br>1 hr                                    |
|                            | Present                     |   | Yes                                  | Yes      | Yes    |  |   |
| Fetal<br>pancreas          | Present                     | Increased glucose penetration   | Yes                                  | Yes      | Yes    | 200  | 1 hr<br>1 hr                                    |
|                            | Present                     |   | Yes                                  | Yes      | Yes    |  |   |
| Tissue<br>from             | Present in some             | Increased glucose utilization   | Yes                                  | Yes      | Yes    | 10 000   | Hr  |
|                            | Absent                      |   | Yes                                  | Yes      | Yes    |  |   |
| Kidney                     | Present                     | Increased glucose utilization   | Yes                                  | Yes      | Yes    | 10 000   | Hr  |
|                            | Present                     |   | Yes                                  | Yes      | Yes    |  |   |
| Fetal<br>pancreas          | Present                     | Increased glucose utilization   | Yes                                  | Yes      | Yes    | 10 000   | Hr  |
|                            | Present                     |   | Yes                                  | Yes      | Yes    |  |   |
| Liver                      | Present                     | Increased glucose utilization   | Yes                                  | Yes      | Yes    | 10 000   | Hr  |
|                            | Present                     |   | Yes                                  | Yes      | Yes    |  |   |

It is table represents the authors' interpretation of the available data. When the question as to demonstration of an effect is answered by yes rather than yes the evidence on which the answer is based is largely indirect in nature.



inadequate utilization of glucose because of insulin lack. Fatty acid release appears to be exquisitely sensitive to hormonal control, particularly to epinephrine [168]. Adrenocorticotrophic hormone and growth hormone have also been implicated in the activation of this release mechanism.

### MECHANISM OF INSULIN ACTION

As yet the diabetic state and insulin have been mentioned only in order to place some emphasis on certain metabolic pathways and tissues. The mechanism of insulin action has been for many years and still is under intense scrutiny in many laboratories. Most investigators agree that in insulin responsive tissues insulin primarily facilitates the conversion of extracellular glucose to intracellular glucose 6-phosphate. Somewhat over ten years ago Colowick, Cori and Stein [169] postulated that insulin directly alters hexokinase activity in the presence of inhibitory pituitary or adrenal components. However Levine and his collaborators [170] measuring the distribution of nonutilizable glucose analogues in eviscerated animals and Ross [171] measuring the penetration of carbohydrates into the anterior chamber of the rabbit eye as well as into the rabbit lens obtained conclusive evidence that the effect of insulin is directly on the cell wall and results in facilitation of the entry of glucose and substances of similar structure across this wall and into the cell. The report of equal levels of hexokinase in normal and diabetic muscle [172] supports this latter mechanism of action. Recent experiments by Morgan et al. on the perfused rat heart [173] and by Kipnis and Cori on the isolated uncut rat diaphragm [174] suggest a dual effect of insulin resulting both in increased penetration of glucose (as measured by the accumulation of free glucose or glucose analogues inside the cell) and in the correction of a decreased intracellular phosphorylating mechanism in the diabetic state. It would also appear from these studies that the effect on permeability is immediate while the effect on phosphorylation requires time and as originally suggested by Cori is sensitive to pituitary and adrenal factors. This double role of insulin is difficult to understand unless phosphorylating and penetrating (or transporting) mechanisms have one or several components in common [175] as may indeed be true. Liver has no demonstrable permeability barrier to glucose [176] yet the diabetic state in liver is characterized by inability to phosphorylate glucose again suggesting some direct effect of insulin on the phosphorylating mechanism itself. The reader is referred to review references for further details and discussions particularly to the recent article by Park and his collaborators [176]. It should however be evident from the preceding paragraph that for the present no categorical statement can or should be issued as to whether the effect of insulin upon glucose utilization primarily results from an action upon

TABLE 3.2 RESPONSIVENESS OF INDIVIDUAL TISSUE TO INSULIN

| Tissue                    | Permissiveness to insulin                              | Major insulin effect  | Effect or lack of effect of insulin |          |         | Concentration of insulin required in microunits/kg | Time required for initiation of effect |
|---------------------------|--|---|-------------------------------------|----------|---------|--|--|
|                           |  |   | In vivo                             | In vitro | In vivo |  |  |
| Muscle                    |  |   |                                     |          |         |  |  |
| Skeletal                  | Present  | Increased glucose uptake in exercised glycolysis. Also increased uptake of amino acids  | Yes                                 | Yes      | Yes     | 10-1000  | Min                                    |
| Liver                     | Present  | Increased incorporation of amino acids into protein   | Yes                                 | Yes      | Yes     | 10   | Min                                    |
| Adipose tissue            | Present  | Increased glucose uptake and oxidation. Increased lipogenesis in the presence of glucose. Also decreased release of fatty acids | Yes                                 | Yes      | Yes     | 2000   | Min                                    |
| Mammary gland (lactating) | Present (sheep)  | Increased glucose uptake  | Yes                                 | Yes      | Yes     | 10-1000  | 1 hr                                   |
| Leukocytes                | Present  | Increased glucose penetration   | Yes                                 | Yes      | Yes     | 10-1000  | 1 hr                                   |
| Lens                      | Present  | Increased glucose transport   | Yes                                 | Yes      | Yes     | 200  | 1 hr                                   |
| Blood/aqueous             | Probably present in some                               | Increased glucose utilization   | Yes                                 | Yes      | Yes     |  | 1 hr                                   |
| Tumors                    | Absent   |   | Yes                                 | Yes      | Yes     |  |  |
| Brain                     | Probably absent  |   | Yes                                 | Yes      | Yes     |  |  |
| Kidney                    | Probably absent  |   | Yes                                 | Yes      | Yes     |  |  |
| Intestinal mucosa         | Absent   |   | Yes                                 | Yes      | Yes     |  |  |
| Erythrocytes              | Probably present but physiologic significance disputed | Increased glycolysis (rat liver)  | Yes                                 | Yes      | Yes     | 10-1000  | 1 hr                                   |
| Liver                     |  | Increased lipogenesis is increased  | Yes                                 | Yes      | Yes     |  |  |

This table represents the authors' interpretation of the available data. When the question as to demonstration of an effect is answered by yes rather than yes, the evidence on which the answer is based is largely indirect in nature.

inadequate utilization of glucose because of insulin lack. Fatty acid release appears to be exquisitely sensitive to hormonal control particularly to epinephrine [168]. Adrenocorticotrophic hormone and growth hormone have also been implicated in the activation of this release mechanism.

### MECHANISM OF INSULIN ACTION

As yet, the diabetic state and insulin have been mentioned only in order to place some emphasis on certain metabolic pathways and tissues. The mechanism of insulin action has been for many years and still is under intense scrutiny in many laboratories. Most investigators agree that in insulin responsive tissues insulin primarily facilitates the conversion of extracellular glucose to intracellular glucose 6-phosphate. Somewhat over ten years ago Colowick, Cori and Stein [169] postulated that insulin directly alters hexokinase activity in the presence of inhibitory pituitary or adrenal components. However, Levine and his collaborators [170], measuring the distribution of nonutilizable glucose analogues in eviscerated animals, and Ross [171], measuring the penetration of carbohydrates into the anterior chamber of the rabbit eye as well as into the rabbit lens, obtained conclusive evidence that the effect of insulin is directly on the cell wall and results in facilitation of the entry of glucose and substances of similar structure across this wall and into the cell. The report of equal levels of hexokinase in normal and diabetic muscle [172] supports this latter mechanism of action. Recent experiments by Morgan et al. on the perfused rat heart [173] and by Kipnis and Cori on the isolated uncut rat diaphragm [174] suggest a dual effect of insulin resulting both in increased penetration of glucose (as measured by the accumulation of free glucose or glucose analogues inside the cell) and in the correction of a decreased intracellular phosphorylating mechanism in the diabetic state. It would also appear from these studies that the effect on permeability is immediate while the effect on phosphorylation requires time and as originally suggested by Cori is sensitive to pituitary and adrenal factors. This double role of insulin is difficult to understand unless phosphorylating and penetrating (or transporting) mechanisms have one or several components in common [175] as may indeed be true. Liver has no demonstrable permeability barrier to glucose [176] yet the diabetic state in liver is characterized by inability to phosphorylate glucose again suggesting some direct effect of insulin on the phosphorylating mechanism itself. The reader is referred to review references for further details and discussions particularly to the recent article by Park and his collaborators [176]. It should however be evident from the preceding paragraph that for the present no categorical statement can or should be issued as to whether the effect of insulin upon glucose utilization primarily results from an action upon

*capacity for glucose* As long as insulin deficiency is not complete compensation may occur with minimal to moderate hyperglycemia and thus minimal glucosuria. With increasing severity of the insulin deficiency higher and higher concentrations of glucose are required to achieve compensation and the effectiveness of this increased blood glucose level is to an increasingly large extent wasted by the occurrence of rapidly mounting urinary losses. Still higher levels of blood glucose can be obtained only at the metabolic cost of profuse hepatic overproduction. It is evident that peripheral underutilization and hepatic overproduction of glucose are intimately related in severe states of insulin deficiency.

Excess hepatic gluconeogenesis entails the concomitant catabolism of fatty acids and protein fatty acids to provide the necessary hydrogen for reduction of pyruvate to glucose protein to contribute carbon to the pyruvate itself either directly or indirectly. Since acetate is unable to contribute a *net* amount of carbon to the reversed glycolytic sequence fatty acids are unable to contribute carbon for gluconeogenesis. Since urea is the major end product of nitrogen metabolism and since acetoacetate is the major intermediary product of hepatic catabolism of fatty acids it follows from the considerations of intermediary metabolism outlined above that the synthesis of one molecule of glucose from two molecules of pyruvate should be accompanied by the accumulation of at least one molecule of acetoacetate and one molecule of urea if the pyruvate was derived from amino acids.

In terms of these concepts persons with mild diabetes with minimal glucosuria (of the order of 5 to 10 gm glucose per day) should not develop ketonacidosis since the excess hepatic catabolism of fatty acids required by this glucose loss does not produce more acetoacetate than can be easily metabolized by the organism. On the other hand diabetic patients who lose 100 to 200 gm glucose per day necessarily catabolize a proportional amount of fatty acids and generate large amounts of ketone bodies. When the production of ketone bodies surpasses the ability of the body to utilize them they accumulate in the blood stream and since their renal threshold is minimal an increasing number is lost in the urine. Since acetoacetate and  $\beta$  hydroxybutyrate have relatively low  $pH$  values they are predominantly excreted as anions in conjunction with cations. Thus sodium and potassium are lost further aggravating the osmotic diuresis already induced by the urinary glucose loss as well as the already existing tendency to metabolic acidosis.

What are the mechanisms whereby the liver obtains the necessary fatty and amino acids for glucose synthesis? Let us first consider relatively recent information concerning adipose tissue metabolism. During carbohydrate deprivation whether due to an absolute glucose lack such as in fasting or in hypoglycemia or to a relative lack such as occurs with insulin deficiency this tissue releases nonesterified fatty acids into the

glucose transport into the cell (translocation) or from an action upon glucose phosphorylation within the cell (transformation). Past experience concerning similar controversies suggests that in time both effects will be accepted and that a connecting link between them will become apparent.

Although a large number of metabolic effects of insulin *in vivo* and *in vitro* can be related directly or indirectly to a primary effect of the hormone on glucose utilization by tissues such as adipose tissue and muscle, there are also some well-documented effects of insulin which cannot be readily explained on this basis alone. These include effects on fatty acid synthesis from acetate by liver [177], effects on hepatic glycogen synthesis [178], effects on amino acid concentration [179] and incorporation into protein [180] by skeletal muscle, effects on hepatic glucose release [181-182] and general effects on permeability of cell membranes for substances as different from glucose as the enzyme aldolase [183]. It has also been suggested that in skeletal muscle insulin primarily affects electric membrane potentials and ionic concentration gradients [184].

Tissues vary markedly in their sensitivity to insulin. Recently it has been shown that adipose tissue may be more sensitive to small concentrations of insulin and to smaller changes in insulin concentrations than many other tissues which have been assayed *in vitro* [185]. Table 3-2 summarizes some of the findings relating to the action of insulin on individual tissues. It is obvious also from this table that certain tissues such as brain are apparently not affected by the presence or absence of insulin, although this does not preclude effects on specific structures of small bulk within these tissues.

### DIABETIC KETOACIDOSIS

Since the primary effect of insulin, or at least one of the primary effects, is to facilitate the utilization of glucose by certain tissues, especially adipose tissue and muscle, lack of insulin results in elevated extracellular fluid glucose levels. Furthermore, the altered hepatic enzyme structure characteristic of the diabetic state, namely decreased glucokinase and increased glucose 6-phosphatase activity, aggravates this tendency to extracellular accumulation of glucose. The resulting hyperglycemia tends to correct *in part* for the defect in glucose utilization, and there has been much debate as to whether this readjustment may compensate for the lack of insulin. In general, in the absence of ketoacidosis it would appear that such a compensation does indeed occur in the diabetic organism, although it is generally assumed that this ability to maintain compensation by hyperglycemia is associated with states of partial rather than complete deficiency of the hormone.

*In terms of overall carbohydrate balance, a major part of the problem which faces the diabetic patient results from the occurrence of massive glucosuria as soon as the hyperglycemia surpasses the renal reabsorptive*

*capacity for glucose* As long as insulin deficiency is not complete compensation may occur with minimal to moderate hyperglycemia and thus minimal glucosuria. With increasing severity of the insulin deficiency, higher and higher concentrations of glucose are required to achieve compensation and the effectiveness of this increased blood glucose level is to an increasingly large extent wasted by the occurrence of rapidly mounting urinary losses. Still higher levels of blood glucose can be obtained only at the metabolic cost of profuse hepatic overproduction. It is evident that peripheral underutilization and hepatic overproduction of glucose are intimately related in severe states of insulin deficiency.

Excessive hepatic gluconeogenesis entails the concomitant catabolism of fatty acids and protein fatty acids to provide the necessary hydrogen for reduction of pyruvate to glucose protein to contribute carbon to the pyruvate itself either directly or indirectly. Since acetate is unable to contribute a net amount of carbon to the reversed glycolytic sequence fatty acids are unable to contribute carbon for gluconeogenesis. Since urea is the major end product of nitrogen metabolism and since acetoacetate is the major intermediary product of hepatic catabolism of fatty acids it follows from the considerations of intermediary metabolism outlined above that the synthesis of one molecule of glucose from two molecules of pyruvate should be accompanied by the accumulation of at least one molecule of acetoacetate and one molecule of urea if the pyruvate was derived from amino acids.

In terms of these concepts persons with mild diabetes with minimal glucosuria (of the order of 5 to 10 gm glucose per day) should not develop ketoacidosis since the excess hepatic catabolism of fatty acids required by this glucose loss does not produce more acetoacetate than can be easily metabolized by the organism. On the other hand diabetic patients who lose 100 to 200 gm glucose per day necessarily catabolize a proportional amount of fatty acids and generate large amounts of ketone bodies. When the production of ketone bodies surpasses the ability of the body to utilize them they accumulate in the blood stream and since their renal threshold is minimal an increasing number is lost in the urine. Since acetoacetate and  $\beta$  hydroxybutyrate have relatively low  $pK$  values they are predominantly excreted as anions in conjunction with cations. Thus sodium and potassium are lost further aggravating the osmotic diuresis already induced by the urinary glucose loss as well as the already existing tendency to metabolic acidosis.

What are the mechanisms whereby the liver obtains the necessary fatty and amino acids for glucose synthesis? Let us first consider relatively recent information concerning adipose tissue metabolism. During carbohydrate deprivation whether due to an absolute glucose lack such as in fasting or in hypoglycemia or to a relative lack such as occurs with insulin deficiency this tissue releases nonesterified fatty acids into the

circulation which bound to albumin are carried to the liver. These fatty acids also serve as metabolic fuels which may be directly utilized by other tissues particularly heart and skeletal muscle. Determination of nonesterified fatty acids in patients with diabetes has shown them to be at least as responsive to insulin as the concentration of blood glucose [186].

Turning to protein, insulin has been shown to increase the incorporation of amino acids into muscle both *in vivo* and *in vitro*. In the absence of insulin and/or carbohydrate the steady state of protein synthesis and breakdown from and to amino acids is altered in favor of protein breakdown. Amino acids rise in the circulation and their hepatic metabolism appears to be roughly proportional to their concentration in blood. Thus both for fatty acids from adipose tissue and for amino acids from muscle deposition or mobilization may be controlled at least in part by availability of insulin, or glucose or both.

Recently several investigators have suggested that insulin also *directly* decreases hepatic glucose release [181-182] although their interpretations have been challenged [187]. The metabolic effects of such a direct interference with hepatic gluconeogenesis would very likely resemble in all points the sequence of events described here. Again as has so frequently been true during the development of endocrine metabolic concepts the controversy does not concern the occurrence of metabolic events but rather the relative importance assigned to individual events both in terms of quantity and in terms of their proximity to the primary site of action of hormones.

In this discussion of the physiologic and biochemical induction of the acute diabetic syndrome no mention has been made of the role played by other endocrines notably the adrenal and pituitary. The 'amelioration' of the diabetic state by hypophysectomy or by adrenalectomy is fully established. It would also seem that cortisone accelerates the efflux of amino acids from muscle [188] and perhaps facilitates their uptake into liver cells [179]. Likewise corticosteroids and possibly pituitary factors such as ACTH or growth hormone accelerate the release of unesterified fatty acids from adipose tissue. It would appear therefore that the adrenal and pituitary glands oppose the effects of insulin on muscle and adipose tissue with regard to amino acid and fatty acid balance respectively. From this it would follow that adrenalectomized or hypophysectomized animals unable to mobilize glucose precursors from the periphery should show decreased gluconeogenesis and therefore less nitrogen loss and a decreased predisposition to ketosis. It should be stressed however that the diabetic adrenalectomized or diabetic hypophysectomized state implies loss of the ability to adapt to either hyperglycemia or hypoglycemia and as the clinician well knows may be more difficult to manage than either endocrinopathy alone.

### Nondiabetic Ketoacidosis

That glucosuria is the prime instigator of the entire sequence of events leading to ketoacidosis is corroborated by observations in two other metabolic states characterized by loss of carbohydrate from the body. The first of these is *the experimental animal in which primary renal loss of glucose has been induced by phlorizin* and which exhibits a sequence of chemical anomalies identical to that noted in diabetes with the exception of hyperglycemia which is replaced by hypoglycemia. Occasionally hereditary renal glucosuria in man may be of such degree that mild ketoacidosis occurs. The second metabolic state to be mentioned here is *bovine ketosis* i.e. carbohydrate loss in milk a condition frequently seen in cows during active lactation. Affected animals usually the optimal milk producers in the herd exhibit classical ketoacidosis with Kussmaul respirations dehydration and collapse. Again hypoglycemia is present rather than hyperglycemia and indeed therapy dramatically effective consists of the intravenous administration of large amounts of 50 per cent glucose. Finally another and well known cause of mild ketoacidosis is the excessive utilization of carbohydrate in the body itself as in severely ill or febrile patients particularly children. Again the metabolic sequence is excessive hepatic gluconeogenesis requiring simultaneous catabolism of fatty acid and protein.

## SUMMARY AND CONCLUSIONS

1 Diabetes mellitus may be defined as a metabolic disorder characterized by hyperglycemia. Although hyperglycemia is not present from birth in the great majority of instances there is excellent evidence to indicate that the potentiality to develop diabetes mellitus is inherited. Best available evidence suggests that the mode of inheritance is a simple recessive one with incomplete penetrance.

2 Genealogies which do not seem consistent with the simple recessive mode of inheritance may be the result of the very high incidence of the diabetic gene combined with inability to recognize its presence prior to the development of hyperglycemia. It has been estimated that in the United States about 5 per cent of the population is homozygous (dd) for the gene determining the susceptibility for diabetes. Available information does not suggest that the major clinical forms of diabetes are genetically distinct the rare syndrome of lipotrophic diabetes being a probable exception.

3 The diabetic manifestations which are more or less readily explained as derived from hyperglycemia have been termed *acute diabetic syndrome* while the *chronic diabetic syndrome* further includes manifestations which develop slowly in the course of the disease and which frequently appear



to have a vascular basis. Evidence derived from the study of experimental diabetes in animals as well as from careful measurements of the mass of the islets of Langerhans in patients suffering from diabetes suggests that the acute diabetic syndrome is the result of absolute or relative deficiency of the hormone produced by the beta cells of the islets of Langerhans i.e. insulin. All the symptoms and signs of the acute diabetic syndrome may be reversed by the administration of insulin. Whether absolute or relative insulin deficiency also accounts for the symptoms and signs of the chronic diabetic syndrome is as yet uncertain.

4. Whereas absolute or relative insulin deficiency is indeed likely to be an important pathogenetic link in the production of the acute diabetic syndrome the mechanism producing this deficiency is as yet completely unknown. Possibilities which have to be considered include the mechanisms of insulin synthesis, storage, release and secretion, transport, destruction and effectiveness at the tissue level. In contrast a great deal of information has accumulated with regard to the pathogenetic pathways linking the acute diabetic syndrome to the postulated insulin deficiency: this information has been derived from the study of animals with experimental forms of diabetes, of diabetic patients and of the action of insulin in the intact organism and in isolated tissues. Much evidence points to the importance of insulin in facilitating the transport and/or metabolic activation of glucose. Indeed most features of the acute diabetic syndrome can be explained as secondary results of decreased glucose utilization by certain tissues among which adipose tissue and muscle probably are of particular importance. The metabolic interrelations upon which this summary statement is based have been discussed in some detail with emphasis upon the fact that the metabolism of carbohydrates, fats and proteins should not be considered separately but as completely and intimately interrelated systems.

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## Chapter 4

### Pentosuria\*

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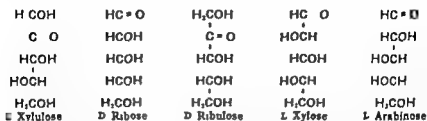
*Howard H. Hiatt*

The first 14 decades which followed the original description by Salowski and Jastrowitz in 1892 of an individual with essential pentosuria [1] produced much information concerning the clinical aspects of this condition but virtually none which cast light on the nature of the biochemical lesion. Before the seventh decade has elapsed however one can predict with confidence that the metabolic defect will have been well characterized. As a result and perhaps even more significantly important information will have been uncovered concerning the operation in normal individuals of a previously unknown pathway of carbohydrate metabolism. Such an elucidation of a normal mechanism by studies of an accident of nature led Garrod [2] 30 years ago to stress the lessons of rare maladies and prompted Harvey [3] 300 years earlier to note that nature is nowhere accustomed more openly to display her secret mysteries than in cases where she shows traces of her workings apart from the beaten path.

A consideration of essential pentosuria requires its separation from other conditions in which a five carbon sugar is excreted in the urine. Essential pentosuria or chronic essential pentosuria is the only member of the group in which a genetic defect accounts for the melituria. It may be further defined as an innocuous condition presumably present from birth in which a relatively constant amount of the pentose L-xylulose appears in the urine. It bears no relationship to diabetes mellitus and has been described almost exclusively in Jews. It is almost surely the result of an impairment in the metabolism of glucuronic acid. It may easily be distinguished from several other situations in which much smaller quantities of certain pentoses other than L-xylulose are found.

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in the urine (Table 4-1). The structure of some of the urinary pentoses follows:



Alimentary pentosuria is the term applied to the excretion of small amounts of arabinose or xylose following the ingestion of unusually large quantities of such fruits as plums, cherries, and grapes and fruit juices [4-6]. Small amounts of D-ribose are often present in urine from healthy

TABLE 4-1 TYPES OF PENTOSURIA

| Type       | Urine pentose                        | Amount excreted gm/24 hr            | Cause                  | Origin of pentose   |
|------------|--------------------------------------|-------------------------------------|------------------------|---------------------|
| Essential  | L-Xylulose                           | 10-40                               | Metabolic error        | D-glucuronic acid   |
| Alimentary | L-Arabinose                          | Less than 0.100                     | Excessive fruit intake | Dietary fruit       |
| Ribosuria  | L-Xylose<br>D-Ribose                 | Up to 0.010                         | Muscular dystrophy     | Nucleic enzymes (P) |
| Normal     | L-Xylulose<br>D-Ribose<br>D-Ribulose | Up to 0.000<br>Up to 0.01<br>Traces |                        |                     |

persons and slightly larger quantities may be found in the urine of some patients with muscular dystrophy [7] presumably as a result of the excessive breakdown of ribose containing nucleotides in degenerating muscle. Finally traces of L-xylulose [8, 9] and of D-ribulose [9] may be found in the urine of normal individuals. It is essential pentosuria with which this review will be primarily concerned.

### HISTORICAL SURVEY

In 1887 Wilham [10] demonstrated that arabinose, the sugar of gum arabic, was a member of a hitherto undescribed class of sugars containing in contrast to the hexoses only five carbon atoms. Shortly thereafter the wood sugar xylose was identified as another member of this group.

Although L-arabinose and L-xylulose are said to be the alimentary pentoses [1, 5] the evidence for this chemical identification is not convincing.

The biologic significance of this class of sugars was strengthened by the report by Salkowski and Jastrowitz of a human being in whose urine a pentose was consistently excreted [1]. This sugar was not fermented by yeast, was optically inactive and yielded an osazone with a melting point of 139°C. The latter observation led the authors to suggest that the sugar was a pentose. Several similar case reports appeared in the German literature during the next decade and in 1906 Janeway [11] in a paper entitled "Essential Pentosuria in Two Brothers" recorded the first instances in an American publication. Although Janeway acknowledged that in many of the previous reports in the literature neurasthenic symptoms were prominent, he stressed that no harmful effects of the disorder were known. He emphasized that the most important responsibility of the physician was to explain carefully to the patient the difference between his ailment and diabetes mellitus and to effect 'the removal of any dietetic restrictions he may have been subjected to'. Although more than a half century has elapsed since Janeway's report, his advice still points out an essential function of any physician confronted by a person with pentosuria. Two years after Janeway's paper in his classic Croonian lectures of 1908 Garrod [12] reviewed 30 recorded cases of essential pentosuria and he assigned this abnormality along with cystinuria, alcaptonuria and albinism to a category which he labeled as inborn errors of metabolism. He stressed the differences between essential pentosuria and the alimentary variety. Garrod noted the incidence of essential pentosuria in Jews and the tendency for the condition to occur in several members of the same family. A prevalent early impression that pentosuria frequently accompanies diabetes mellitus [13] has not been borne out.

Much of the present knowledge concerning the clinical aspects of pentosuria, its genetic transmission and the nature of the urinary sugar is derived from the careful observations of Enklewitz and Lasker [14-20]. More than half of their 70 subjects with pentosuria were followed for periods in excess of 16 years. Fifty years following the first description of pentosuria, Derivaux [21] was able to collect 163 case reports from the literature and at the time of a recent review of the subject this figure exceeded 200 [22].

For many years controversy existed concerning the nature of the sugar in pentosuric urine. The first chemical identification was that of Neuberg [23] who in 1900 reported that the sugar present in the urine of one case was racemic arabinose. Five additional individuals with arabinosuria were subsequently described by Cammidge and Howard [24] and single similar cases were noted by a number of authors including Aron [25], Luzzatto [26] and Schüler [27]. Zerner and Waltuch [28] in 1913 presented convincing evidence that the urinary pentose was optically active and that it was not arabinose. The following year Levene and LaForge showed that

the urinary sugar in a case of pentosuria was L-xylose.<sup>3</sup> [29] Their identification was based on the following observations:

1. An osazone of the urinary sugar had a melting point of 160 to 163°C when it was mixed with an osazone of D-xylose the melting point was increased by 10°.

2. The initial optical rotation of the osazone was lower than the equilibrium rotation, this is characteristic of xylosazone but not of arabinosazone.

3. The foregoing observations indicated that the urinary pentosazone was a xylosazone. On the basis of the optical rotation of the urinary sugar ( $\alpha_D^{20} = +33.1^\circ$ ) the character of its *p*-bromophenylhydrazone and its behavior in oxidation experiments. Levene and Torge concluded that the urinary pentose could only have been L-xylose.

In the same year Zerner and Waltuch [30] isolated the sugar from the urine of two patients with pentosuria and also concluded that it was L-xylose. A similar conclusion concerning the pentose in their cases was reached by Hiller [31], Greenwald [32] and Finkewitz and Lasker [15]. Some of the early reports of arabinosuria were subsequently corrected. For example a patient of Solis Cohen was first described in 1909 as having arabinosuria [33]. At that time the author assumed from previously reported cases that arabinose was the urinary sugar excreted in pentosuria. However the patient was followed for almost three decades and in a follow-up report which appeared in 1936 [34] the same author indicated that he had identified the urinary sugar as L-xylose and that his earlier impression had been in error. Similarly a case reported as one of arabinosuria in 1913 [20] was reexamined 40 years later; paper chromatography of his urine revealed the major sugar component to be L-xylose and no evidence of arabinose was found [35].

The question of arabinosuria was considered at length in 1950 by Lasker [19] who indicated that she had identified L-xylose in the urine of 72 individuals with pentosuria but had never seen a patient with arabinosuria. She further stated that no case of arabinosuria had been reported since 1928 and that since in early reports the arabinose was always identified by the same method this impression may have been in error. Recent studies with paper chromatographic techniques provide further corroboration that L-xylose is the only sugar excreted in substantial amount in pentosuria. In addition to L-xylose a small quantity of L-arabitol has been isolated from the urine of one pentosuric patient [36]. Other reports that pentosuric patients excrete more than one pentose [35, 37] will be considered below.

<sup>3</sup> The early reports of D-xylosketose excretion were published prior to the adoption of the current practice of classifying sugars according to their structural relation to D- and L-glyceraldehyde. Throughout this review the present convention will be followed regardless of the terminology applied in the original reports.

The constancy of the amount of urine pentose excreted by individuals with pento uria has long been recognized. Enklewitz and Lasker [16] found that the excretion in five adults varied from 1.1 to 3.7 gm per 24 hr but that the daily variation in any given subject never exceeded 0.9 gm. The excretion is independent of dietary variations: early reports by Janeway [11] and Kiercker [13] that the amount of urinary pentose can be altered by changes in dietary nucleic acid or protein have not been substantiated. Margolis noted a marked increase in pentose excretion in pentosuric subjects following aminopyrine ingestion [38]. When Enklewitz and Lasker observed a similar stimulation not only following the intake of certain other drugs including borneol, antipyrine and menthol but also after the administration of glucuronic acid [16] they attributed the effect of the drugs to their glucuronogenic action. The metabolic interrelations of glucuronic acid and L-xylulose, however, remained obscure and there was no experimental evidence for Everett's postulate that an abnormal enzyme system existed in pentosuric patients which decarboxylated glucuronic acid to L-xylulose [39]. With the recent identification of L-xylulose as an intermediate in the metabolism of glucuronic acid [40-41] and the elucidation of the reactions involved in the further metabolism of L-xylulose [42-43] much progress has been made toward an understanding of the biochemical abnormality in pentosuria. Much of the present concept stems from the careful studies of Touster and his associates [40-43]. Further important contributions have come from the laboratories of Ashwell [44-46], Burns [47], Lehninger [48] and others. Not only have these recent studies permitted an insight into the biochemical aberration in pentosuria but they have also provided important information concerning the operation of the glucuronic acid oxidation pathway in normal individual.

## PENTOSE METABOLISM IN MAN

Several five carbon sugars are known to be present in the human organism. Some such as ribose and deoxyribose are present as part of more complex substances including the nucleic acids and certain coenzymes. Other, including D-xylulose, ribulose and L-xylulose, are intermediates in metabolic pathways and normally are not detectable in body fluids or are present only in trace quantity. Finally, there are those which are not known to be synthesized by man but which occasionally may be ingested and thereafter are excreted in the urine. These include arabinose and xylose.

In recent years much information has come to light concerning previously obscure pathways of carbohydrate metabolism (for a summary see [49]). Certain of these schemes involve pentoses as key intermediates. A consideration of these pathways is essential to understanding of the



latter may be isomerized to ribose 5 phosphate. In this series of reactions for every molecule of pento = phosphate synthesized from hexose phosphate two molecules of triphosphopyridine nucleotide (TPN) are reduced to TPNH. Much evidence has accumulated indicating that the conversion of the coenzyme from its oxidized to its reduced form is as important a function of these reactions as is pentose production [49]. This conclusion is based on the increasing evidence for the TPNH requirement of a variety of reductive synthetic reactions and on observations which suggest that the oxidative reactions of the pentose phosphate pathway are the principal means available to the cell for TPN reduction.

Ribose may also be produced nonoxidatively from hexose phosphate via the transketolase and transaldolase reactions. In these reactions the first two carbon atoms of one molecule of fructose 6-phosphate may be cleaved and condensed with a molecule of triose phosphate under the influence of the enzyme transketolase. This results in the formation of one molecule of xylulose 5-phosphate and one of erythrose 4 phosphate. The latter together with another molecule of fructose 6-phosphate may then participate in the transaldolase reaction resulting in the production of a molecule of sedoheptulose 7 phosphate and one of triose phosphate. In another reaction catalyzed by transketolase these products may undergo conversion to xylulose 5 phosphate and ribose 5-phosphate. The xylulose 5 phosphate may be epimerized to ribulose 5 phosphate which as already noted can be isomerized to ribose 5 phosphate. Thus in this series of reactions there is no net loss of carbon and two molecules of hexose phosphate and one of triose phosphate may be converted to three molecules of pentose phosphate. Available data indicate that in animals [51-52] and in man [53] ribose is normally synthesized from hexose by way of both the oxidative and the nonoxidative reactions of the pentose phosphate pathway. The fact that thiamine pyrophosphate is a cofactor for the enzyme transketolase [54] accounts for the block in ribose synthesis by way of the nonoxidative sequence of reactions in the thiamine deficient animal [55].

In contrast to the oxidative reactions which afford only a mechanism for pentose production from hexose, the transketolase-transaldolase sequence is reversible and provides a means for the interconversion of hexose and pentose [56]. Evidence has been presented demonstrating that the nonoxidative reactions mediate the conversion of ribose to hexose in man [57]. It has also been shown that ribose may participate directly in ribotide [55] and nucleic acid [58] synthesis. Thus mechanisms exist for the disposition of any ribose released in nucleic acid or coenzyme breakdown.

Small quantities (up to 2.8  $\mu$ moles per 24 hr per kg body weight) of ribose have been reported in normal human urine [7]. This presumably represents either newly synthesized ribose or pentose released from





The elucidation of the reactions involved in this pathway of metabolism provides insight not only into the origin of the L-xylulose found in trace amount in normal urine but also into the probable site of the defect in individuals with chronic essential pentosuria. It also provides an explanation for the hitherto poorly understood increase in L-xylulose excretion by pentosuric subjects given glucuronic acid.

The significance of the glucuronic acid oxidation pathway in human metabolism appears relatively minor. This view is supported by the fact

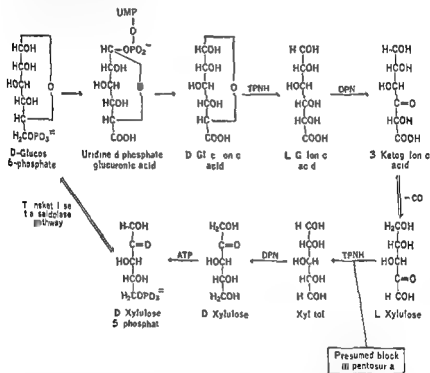


Fig. 4. The glucuronic acid oxidation pathway.

that individuals with pentosuria in whom this pathway is presumably blocked suffer no ill effects from the abnormality. Although the reactions of the glucuronic acid oxidation pathway together with those of the pentose phosphate pathway provide a potential mechanism for ribose biosynthesis from hexose (i.e. hexose phosphate  $\rightarrow$  glucuronic acid  $\rightarrow$  xylulose  $\rightarrow$  xylulose phosphate  $\rightarrow$  ribose phosphate) animal studies indicate that little ribose is normally produced by way of this sequence of reactions [66]. In most mammalian species the early reactions of the pathway are required for the production of L-gulononic acid a precursor of ascorbic acid [67]. In primates and the guinea pig however

nucleic acids or coenzymes which escapes further metabolism. It has been asserted that ribose excretion is slightly but significantly increased in patients with myopathies presumably because of the increased breakdown of ribose-containing compounds in diseased muscle [7] but this observation has not met universal acceptance [9]. A metabolite of histamine, imidazoleacetic acid, is excreted by the rat [60-61] and by man [63] in part as the ribide. Ribulose, a trace constituent of normal urine [9] is presumably excreted following hydrolysis of the phosphate ester which is an intermediate in the pentose phosphate pathway.

The mechanism involved in the synthesis of deoxyribose, the sugar component of deoxyribonucleic acid, has not yet been completely elucidated but at least a portion of it appears to be synthesized via direct reduction of the ribose molecule at the ribonucleotide level [62].

### THE GLUCURONIC ACID OXIDATION PATHWAY

The pentoses, L-xylulose and D-xylulose and the pentitol, xylitol, are intermediates in the glucuronic acid oxidation pathway (Fig. 4.2). The reactions leading to the production of D-glucuronic acid *in vivo* have not been completely elucidated but its carbon skeleton is known to originate in glucose [63-65] and the production of D-glucuronic acid from uridine diphosphate glucuronic acid has recently been demonstrated *in vitro* [66]. As has been noted the studies of Touster [40-43], Ashwell [44-46], Burns [47] and Lehninger [48] and their associates have provided much information concerning the further metabolism of D-glucuronic acid. A reductive reaction involving 1PNH as a cofactor results in the conversion of D-glucuronic acid to L-gulononic acid. L-Gulonolactone has been shown to be an intermediate in the synthesis of ascorbic acid in most animal species but in primates and in the guinea pig, this transformation cannot take place. L-Gulononic acid, however, may in all animal species examined be oxidized to the 3-keto derivative which in turn is decarboxylated to L-xylulose [46]. In the latter reaction the atom corresponding to the sixth carbon atom of the parent glucose molecule is oxidized to CO<sub>2</sub>. Carbon atom 1 of L-xylulose is derived from the fifth carbon atom of glucose. Touster has prepared two enzymes from guinea pig liver, one of which catalyzes the reduction of L-xylulose to the sugar alcohol, xylitol, and the other of which promotes the oxidation of xylitol to D-xylulose [42-43]. The reactions require 21NH and diphosphopyridine nucleotide (DPN) respectively. It is noteworthy that in contrast to other schemes of intermediary carbohydrate metabolism in which only phosphate esters participate, these reactions involve the free sugars. Hickman and Ashwell [49] have described a kinase for D-xylulose in mammalian liver. D-Xylulose 5-phosphate will be recognized as an intermediate in the pentose phosphate pathway and may of course be converted to ribose 5-phosphate and to hexose phosphate.

tography of deionized urine from several pentosuric subjects. Since one substance which appeared in all the urines examined by Barnes and Bloomberg had a chromatographic mobility similar to that of xylose, some of the author's recent unpublished observations seem pertinent. Following the procedure of Barnes and Bloomberg the author too found a xylose-like component in the urine of two pentosuric subjects. This material was eluted from paper chromatograms and was found by Ashwell [73] to behave like L-xylose in several enzymatic reactions. However, this substance was not present in pentosuric urine subjected to paper chromatography *without* prior exposure to ion exchange resins. In addition, normal urine to which only L-xylulose was added and which was then deionized and chromatographed on paper also exhibited the xylose spot. Accordingly, the author has concluded that the appearance of L-xylose in chromatograms of pentosuric urine is an artifact attributable to the preparative procedure.

### *Site of the Defect*

Although Enklewitz and Lasker [16] demonstrated more than twenty years ago that glucuronic acid administration greatly enhanced the excretion of L-xylulose, it was not until the recent elucidation of the glucuronic acid oxidation pathway that a plausible hypothesis could be advanced concerning the biochemical abnormality in pentosuria. In our attempts to define the metabolic lesion it will be helpful to cite existing evidence which supports the following propositions:

1. Glucuronic acid is a direct precursor of the urinary pentose.
  2. Glucuronic acid is incompletely metabolized in pentosuric individuals.
  3. Although the pentosuric person can convert glucuronic acid to L-xylulose, a block exists in the further metabolism of the pentose.
- Touster and his associates [40] confirmed the observation of Enklewitz and Lasker that the ingestion of glucuronolactone is followed by an increased excretion of L-xylulose by pentosuric persons and they postulated that the conversion of glucuronolactone to xylulose is direct. They substantiated this hypothesis by two studies in a pentosuric subject [41]. First, they demonstrated that the administration of glucuronolactone labeled with  $C^{14}$  in its sixth carbon atom (the carboxyl carbon) was followed by the excretion of nonisotopic L-xylulose. Second, they showed that the ingestion of glucuronolactone  $1-C^{14}$  was followed by the urinary excretion of heavily labeled pentose, the isotope of which was predominantly in the fifth carbon atom. These data indicate that the carboxyl carbon of glucuronic acid is lost in the conversion to L-xylulose but that the rest of the molecule is preserved intact. Touster also demonstrated that some normal human beings and guinea pigs excrete traces of L-xylulose and that this excretion is slightly augmented following glucurono-

ascorbic acid cannot be synthesized. Thus it is possible that in man the glucuronic acid oxidation pathway functions only to return a portion of glucuronic acid carbon to the mainstream of carbohydrate metabolism.

### *Effects of Drugs*

The basis for the effect of drugs and hormones on the glucuronic acid oxidation pathway remains a fascinating but largely unexplored area. Margolis's early observation that aminopyrine stimulates the excretion of L-xylulose by individuals with pentosuria [38] was followed by reports indicating that this drug and others such as Chloretone and barbital stimulate free glucuronic acid [68] and ascorbic acid [69] excretion in animals. The drug effect is apparently not related to any known detoxification mechanism since barbital is excreted unchanged. Hormonal involvement in this process is indicated by the observation of Burns and his associates that the administration of Chloretone or barbital to hypophysectomized rats is not followed by an increase in ascorbic acid excretion [68]. It has also been observed that thyroid hormone stimulates pentose excretion in the rat [70] and that the increase in pentose excretion which results from exposure to low temperatures is prevented by the administration of thyroid blocking agents [71]. An increase in L-xylulose excretion by normal human beings given triiodothyronine has been reported [72].

## THE BIOCHEMICAL ABNORMALITY IN ESSENTIAL PENTOSURIA

### *Nature of the Urinary Sugar*

As has been pointed out, there is now conclusive evidence that the urinary sugar excreted by pentosuric persons is L-xylulose (L-xylulose and L-threopentulose). In the light of present knowledge one must conclude that the early purported demonstrations of urinary arabinose either were in error because of deficiencies in the experimental methods employed or else were concerned with patients who do not fit into this category. L-Xylulose is excreted in fairly constant amount, ranging between 1.0 and 3.5 gm per 24 hr. The excretion is increased following the intake of glucuronic acid and of certain drugs, but it is unaffected by diet and is not altered by insulin administration.

Much smaller quantities of other sugars in addition to L-xylulose have been reported in pentosuric urine. Touster and his associates [36] found arabitol and suggested that this sugar alcohol may be derived from L-xylulose reduction. Wolf, Cohn, and Devaney [37] observed a second unidentified component following chromatography of pentosuric urine and Barnes and Bloomberg [35] found small amounts of several substances in addition to large quantities of L-xylulose following chroma-

first 12 hr, 57 per cent of the administered radioactivity appeared in the urine of the normal individual and 76 per cent in that of the pentosuric person. Thus the total  $C^{14}$  excretion in urine and expired air of the normal and pentosuric subjects during the intervals measured was in excess of 73 and 79 per cent respectively.

### *Further Isotope Experiments*

The foregoing study strongly supports the view that glucuronic acid metabolism is impaired in pentosuria. Some information concerning the site of the defect may be deduced from the results of a published experiment [74] which will be briefly summarized. Using a "ribose-trapping" technique suggested by the observations of Tabor and Hayaishi [60] and of Karjala [61] that imidazoleacetic acid is excreted in the urine in part as a riboside, the author has demonstrated ribose synthesis from hexose in man by way of the oxidative and nonoxidative reactions of the pentose phosphate pathway [53]. If the glucuronic acid oxidation pathway were operative in normal individuals, it would afford a mechanism for the conversion of glucuronolactone carbon to ribose in the following fashion: glucuronolactone  $\longrightarrow$  L-xylulose  $\longrightarrow$  D-xylulose  $\longrightarrow$  D-xylulose 5-phosphate  $\longrightarrow$  ribose 5-phosphate. (For an outline of the reactions involved, see Fig. 4.2.) Thus the administration of imidazoleacetic acid and of glucuronolactone uniformly labeled with  $C^{14}$  should be followed by the excretion of imidazoleacetic acid riboside containing ribose- $C^{14}$ . On the other hand, if this pathway were blocked in pentosuria at a site beyond L-xylulose, then a pentosuric subject might be expected to excrete radioactive L-xylulose but nonisotopic riboside ribose. (Since there is no reason to postulate an impairment in the pentose phosphate pathway in pentosuria, the synthesis and excretion of the riboside ribose should proceed normally.) The results of such an experiment are described in Table 4.2. The significant  $C^{14}$  incorporation in the urinary riboside ribose of the normal subject is consistent with the conversion of glucuronolactone to ribose by way of the reactions of the glucuronic acid oxidation and the pentose phosphate pathways (Fig. 4.2). The pentosuric subject excreted ribose in an amount comparable to that excreted by the normal individual, but the virtual absence of radioactivity indicates that it was derived from sources other than glucuronolactone. The large quantity of isotope in the urinary L-xylulose is in agreement with Touster's demonstration that glucuronic acid is a direct precursor of the pentose. This experiment not only provides information concerning the impairment in pentosuria, but also helps to establish the concept that the glucuronic acid oxidation pathway is operative in normal individuals.

It must be emphasized that while these experiments provide strong circumstantial evidence that an abnormality in the glucuronic acid

lactone ingestion. These observations were considered to strengthen the concept that L-xylose is a metabolic intermediate in normal subjects.

A recent unpublished study may be cited in support of the hypothesis that a defect in glucuronic acid metabolism exists in pentosuria. Let us postulate that a pentosuric person can remove a carbon atom 6 of glucuronolactone with unimpaired efficiency but that he is incapable of metabolizing the remainder of the carbon chain. Then following the administration of glucuronolactone uniformly labeled with  $C^{14}$  there should be six

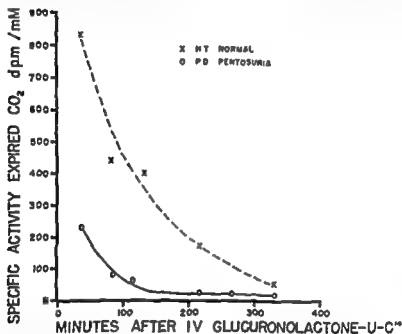


Fig. 4-3  $C^{14}O_2$  excretion in the expired air following the intravenous administration of glucuronolactone uniformly labeled with  $C^{14}$  to a normal subject and to a pentosuric individual.

times as much  $C^{14}O_2$  in the expired air of a normal person as is present in that of a pentosuric person. These expectations were fulfilled in a study, the results of which are depicted in Fig. 4-3. In this experiment 5 microcuries of glucuronolactone uniformly labeled with  $C^{14}$  (obtained through the generosity of Dr. N. Artz, Corn Products Refining Co., Argo, Ill.) was given intravenously to a 16-year-old male with essential pentosuria and to a 22-year-old female with no abnormality of carbohydrate metabolism. Samples of expired air were collected at intervals for 6 hr thereafter and were analyzed for  $CO_2$  and for radioactivity. In this period of time 16 per cent of the administered  $C^{14}$  appeared in the expired air of the normal subject and 2.6 per cent in that of the pentosuric subject—almost exactly the sixfold difference that was predicted. In the

two normal individuals a sugar which was identified as xylulose by paper chromatographic techniques (Flynn [79] had previously demonstrated xylulose in pentosuric plasma by chromatography). After glucuronolactone administration the serum xylulose level in a pentosuric subject of Bozian and Touster reached 11 mg per 100 ml. Simultaneous with the Bozian and Touster study Freedberg, Feingold and Hunt [80] used the specific enzymatic assay of Hickman and Ashwell [81] to measure L-xylulose levels in the serum and urine of several non Jewish controls, three pentosuric persons and four close relatives of pentosuric persons (Table 4.3). From the latter work several points merit emphasis. Fasting serum L-xylulose levels in all three pentosuric persons were in excess of 1 mg per 100 ml while in the serums of all but one of the other individuals no L-xylulose was detectable in the fasting state. In all but two subjects D-glucuronolactone led to a rise in serum L-xylulose levels with a peak generally reached within 1 hr of feeding. The rise in serum pentose level was accompanied by a marked increase in urinary pentose in the pentosuric persons and by the appearance of urinary pentose in all other subjects tested. The serum levels of four heterozygotes (two D and E in Table 4.3 are mothers of pentosuric persons, a third F is a brother of three persons with pentosuria, G is the child of a pentosuric mother) were higher than those of the normal subjects and the urine levels in the two heterozygous subjects tested were considerably greater than those in the control subjects. Assuming normal glomerular filtration rates for the 8 subjects one can calculate that virtually all L-xylulose entering the renal glomeruli appears in the urine of normal as well as of heterozygous and pentosuric subjects. Thus a defect in renal tubular reabsorption of L-xylulose appears to exist in all subjects but it is clearly not the metabolic error which distinguishes the pentosuric from the normal person.

## CLINICAL CONSIDERATIONS

### *Manifestations*

Pentosuria can be classified with those inborn errors of metabolism in which no disturbance of function has been demonstrated to result from the genetic abnormality. Indeed the most frequent difficulty encountered by pentosuric subjects is consequent to a mistaken diagnosis of diabetes mellitus and the institution thereafter of dietary and insulin therapy. A number of reports in the literature indicate that only after an episode of insulin induced hypoglycemia is the correct diagnosis been made. No abnormality of glucose metabolism is demonstrable except in those rare instances in which pentosuria and diabetes mellitus coincide. Several authors have commented on the frequency and severity of psychologic disturbances encountered in subjects with pentosuria.



oxidation pathway exists in pento uria the enzymatic defect has yet to be demonstrated. Since pento uria is a benign as well as an uncommon disturbance and further since the enzymes involved in L-xylulose metabolism have thus far been found only in mammalian liver opportunities to define the abnormal site with precision will be exceedingly sparse. Until such a demonstration conclusions must be tentative.

Knox pointed out that impaired L-xylulose reabsorption by the renal tubule could be the defect present in pento uria [22]. An experiment which was cited in support of this possibility is that of Enklewitz and Lasker [14] who gave 5 gm L-xylulose by mouth to a pento uric subject

TABLE 4<sup>2</sup> URINARY L-XYLULOSE AND RIBOSE IN NORMAL AND PENTOSURIC SUBJECTS GIVEN IMIDAZOLFACTIL ACID (ImAA) AND D-GLUCURONOLACTONE UNIFORMLY LABELED WITH C<sup>14</sup>

|   | Normal person     | Pentosuric subject |
|---|-------------------|--------------------|
| D-glucuronolactone administered 1 count/min<br>μmoles | $1.5 \times 10^4$ | $7.80 \times 10^4$ |
| ImAA hydrochloride administered μmoles                | 170               | 180                |
| Urinary L-xylulose (0-10 hr) μmoles                   | 1000              | 1000               |
| Relative molar activity counts/min/μmole              | 0.1               | 3900               |
| Total C <sup>14</sup> content counts/min              | 100               | $3.30 \times 10^4$ |
| Per cent administered C <sup>14</sup>                 | 0.00              | 1.3                |
| Ribose from urinary ImAA riboside (0-10 hr)<br>μmoles | 25                | 09                 |
| Per cent administered ImAA                            | 14                | 21                 |
| Relative molar activity counts/min/μmole              | 33                |                    |
| Total C <sup>14</sup> content counts/min/μmole        | 9100              | 110                |
| Per cent administered C <sup>14</sup>                 | 0.008             | 0.002              |

<sup>2</sup>Significance doubtful because of small quantity of radioactivity

and found only 0.5 gm 'additional' L-xylulose in the urine. This suggested metabolism of the pentose. However in the absence of information concerning the efficiency of L-xylulose absorption from the human gastrointestinal tract the Enklewitz and Lasker experiment must be interpreted with caution. Indeed there are published studies which indicate that although parenteral administration of L-xylulose to animals is followed by extensive metabolism [40-75-76] the pentose orally administered is not glycogenic in rats [77]. Moreover even if considerable absorption takes place the possibility exists that the absorbed pentose might inhibit endogenous L-xylulose production by way of a feed back mechanism.

Following the publication of Knox's review two studies appeared which convincingly exclude the renal abnormality hypothesis. Bozian and Touster [78] found in the plasma of a pentosuric subject but not of

but a causal relationship between such disturbances and the error in carbohydrate metabolism has not been established and the suggestion is often made that at least some neurotic complaints may be related to the conflicting medical opinions to which many patients have been subjected. Lasker has followed 40 pentosuric individuals for periods in excess of 16 years and has found no decrease in life expectancy as compared with normal individuals [20]. Some typical clinical considerations may best be presented by citing a hitherto unpublished report of a case of pentosuria first diagnosed in 1938.

P. D., a 16-year-old high school sophomore, was first seen in the Out Patient Department of the Beth Israel Hospital in February, 1958. One year previously a physician had found a reducing substance in the patient's urine and had placed him on a low carbohydrate diet. Despite faithful adherence to this regimen the urine continued consistently to show a 1+ reaction in the Benedict's test. For this reason his physician suggested instituting insulin therapy, and the patient's father brought him to the Beth Israel Hospital to seek additional opinion. The patient had never noted polydipsia, polyuria, or polyphagia, and during the 6 months prior to admission he had gained 8 lb in weight. The father could not recall a single negative urine sugar test during this period, but at no time did he find more than a 1+ reduction. There was a family history of diabetes mellitus in a paternal great uncle, but not of other known disturbances of carbohydrate metabolism. The patient's parents were Austrian born Jews who were not consanguineous, and whose forebears had come from Poland. His past medical history was not contributory, except in two important respects. Ten years and six years previously the patient had been seen in the Pediatric Clinic of the Beth Israel Hospital for upper respiratory infections. Urinalyses carried out on both occasions were recorded as having shown a 1+ positive Benedict's test. In addition, he had frequently been seen by psychiatrists during the previous decade because of problems of behavior. During the year prior to admission his emotional disturbances were apparently magnified by his concern over his condition and also by his resentment at the dietary restrictions to which he had been subjected.

Physical examination revealed a well-developed, well-nourished young male who appeared in good health. Vital signs were normal, and no significant abnormalities were found on examination. Laboratory studies revealed a normal hemogram, a 2 hr postprandial blood sugar of 98, and a urinalysis that was normal except for a 1+ positive test for a reducing substance. The urine, however, did not give a positive reaction with Testape (an enzyme-impregnated paper which reacts specifically with glucose). The urine sugar was shown to be L-xylulose by preparation of the osazone and by paper chromatography in a butanol-ethanol-water (50:10:40) with authentic L-xylulose as a standard, followed by staining with the orcinol-trichloroacetic acid reagent [8]. A glucose tolerance test (50 gm glucose by mouth) showed a fasting blood sugar of 80 mg per 100 ml and blood glucose levels at 30, 60, 120, 180, and 240 min of 120, 110, 95, 80, and 60 mg per 100 ml, respectively. The patient's 24 hr urinary excretion of L-xylulose was found to be 2 gm, and this rose to 4 gm during the 24 hr following the ingestion of 5 gm of D-glucuronolactone. The patient and his parents were reassured concerning the benign nature of this disturbance in carbohydrate metabo-

TABLE 4-3 SERUM AND URINE L-XYLULOSE LEVELS BEFORE AND AFTER ORAL ADMINISTRATION OF D-GLUCURONOLACTONE

| Subject               | Amount of<br>glucuronolactone<br>gm | Serum L-Xylulose     |                      |                                       | Urine L-Xylulose |                  |                                      |
|-----------------------|-------------------------------------|----------------------|----------------------|---------------------------------------|------------------|------------------|--------------------------------------|
|                       |                                     | Fasting<br>mg/100 ml | Maximal<br>mg/100 ml | Time after<br>glucuronolactone<br>min | Fasting<br>mg/hr | Maximal<br>mg/hr | Time after<br>glucuronolactone<br>hr |
| Pentotouric subjects  |                                     |                      |                      |                                       |                  |                  |                                      |
| A                     | 5                                   | 1 -                  | 7 -                  | 60                                    | 106              | 30               | 1-2                                  |
| B                     | 10                                  | 1 1                  | 2 1                  | 60                                    | 48               | 41               | 1-2                                  |
| C                     | 10                                  | 1 -                  | 14 -                 | 30                                    |                  | 40               | 1 -                                  |
| Pentotouric relatives |                                     |                      |                      |                                       |                  |                  |                                      |
| D                     | 5                                   |                      | 1 6                  | 60                                    |                  |                  |                                      |
| E                     | 5                                   |                      | 3 9                  | 30                                    |                  |                  |                                      |
| F                     | 5                                   | 0 14                 | 0 41                 | 30                                    |                  | 41               | 0 1                                  |
| G                     | 5                                   |                      | 1 41                 | 30                                    | 0 1              | 41               | 0 1                                  |
| Control subjects      |                                     |                      |                      |                                       |                  |                  |                                      |
| H                     | 5                                   |                      |                      |                                       |                  | 9                | 0-1                                  |
| I                     | 5                                   |                      |                      |                                       |                  | 22               | 0-1                                  |
| J                     | 5                                   |                      | 0 42                 | 90                                    |                  | 10               | 0-1                                  |
| K                     | 25                                  |                      | 0 29                 | 90                                    | 0 6              | 13               | 0 1                                  |
| L                     | 25                                  |                      | 0 15                 | 60                                    | 0 3              | 18               | 1-2                                  |

Less than 0.1 mg per 100 ml

has a characteristic mobility ( $R_f = 0.26$ ) which exceeds those of other commonly observed urinary sugars [82] and gives a red color on staining with the orcinol trichloroacetic acid reagent [83]

3 *Cysteine-carba ole test* [84]

4 *Behavior of Osazone* [29] The phenylosazone of L-xylulose has a melting point of about 160 C. When it is mixed with the osazone of D-xylulose the crystalline appearance is radically altered (Fig 4-4) and the melting point rises approximately 40

## GENETIC CONSIDERATIONS

Estimates of the incidence of pentosuria vary; the most widely accepted figure 1 in 50 000 is derived from studies of applicants for life insurance examinations [77]. The age at which the diagnosis has first been made varies from 2 to 61 years, but it is likely that pentosuria is present from birth. The earlier reports that pentosuria occurs predominantly in males [12] is not substantiated by studies of the families of pentosuric persons where the incidence seems evenly divided between the sexes. The thesis that pentosuria behaves as a simple recessive anomaly was first proposed by Garrod [12] and has been amply confirmed. Lasker, Enklewitz and Lasker [18] found 37 instances of pentosuria in 20 families. They set forth the following evidence to support their view that pentosuria behaves as a recessive characteristic:

1 Despite its rarity in the general population they found 10 new cases among 34 brothers and sisters of pentosuric propositi.

2 Most cases are among Jews. (A recent report demonstrating with chromatographic techniques pentosuria in two sisters of Lebanese descent [95] is the first instance of proved xylulosemia in non Jews.)

3 The presence of the abnormality in the children of 10 families in which neither parent had pentosuria indicates that the condition is recessive. In the 10 sibships 16 of 38 members had pentosuria. Deducting the 8 propositi leaves 8 of 30 members with pentosuria, a figure consonant with the Mendelian ratio for a recessive characteristic when both parents are heterozygous.

4 One of their subjects was a child of first cousins (one of the parents had pentosuria).

Reference has already been made to studies of serum and urinary L-xylulose [80] which are now continuing in an effort to obtain more information concerning the transmission of the pentosuria gene. Thus far a defect in L-xylulose metabolism after (but not before) glucuronolactone administration has been shown in two mothers of individuals with pentosuria, in a man who is one of five siblings three of whom have been shown to have pentosuria, and in the son of one of the latter siblings with pentosuria.

lism and specifically concerning the absence of any relationship of his condition to diabetes mellitus. He was returned to an unrestricted diet and according to his parents within a month many of the behavioral disturbances which had been present during the previous year were greatly diminished.

### *Diagnostic Measures*

A diagnosis of pentosuria should be suspected in any person and particularly in a Jewish person, who has none of the symptoms of diabetes mellitus but in whose urine a small quantity of a reducing substance is consistently found. This possibility is strengthened in those instances in which the urine does not give a positive test with any of the enzymatic methods specific for glucose. The measures which have proved most

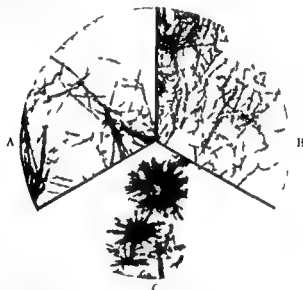


Fig. 4-4. Osazone crystals of *L*-xylulose (A), *D*-xylulose (B) and a mixture of A and B (C). (By permission of H. D. Barnes et al. [35].)

useful in establishing the diagnosis of pentosuria may be summarized as follows:

1. *Reduction of Benedict's reagent at low temperature* [15]. *L*-Xylulose is a strong reducing substance and in contrast to glucose and most other urinary sugars will reduce Benedict's reagent at 55°C in 10 min or at room temperature in 3 hr. (Fructose will also reduce Benedict's reagent at low temperature.)

2. *Paper chromatography*. On paper chromatography *L*-xylulose can be readily distinguished from other sugars. For example, with a mixture of *n*-butanol, ethanol, and water (50:10:40) as the solvent, *L*-xylulose

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## SUMMARY

1 Essential pentosuria is an inborn error of metabolism in which 1.0 to 4 gm of the pentose L-xylulose is excreted in the urine each day. It is a benign disturbance which occurs almost solely in Jews and which behaves genetically as an autosomal recessive characteristic.

2 This disorder bears no relation ship to diabetes mellitus and is easily distinguished from several other varieties of pentosuria in which milligram quantities of a number of pentoses other than L-xylulose appear in the urine.

3 Essential pentosuria is almost surely the result of a defect in the glucuronic acid oxidation pathway. In this route of carbohydrate metabolism the carboxyl carbon atom of D-glucuronic acid is removed in a series of reactions giving rise to the pentose L-xylulose. The latter may then be converted to its stereoisomer D-xylulose which in turn may be phosphorylated. D-xylulose 5-phosphate may participate in reactions of the pentose phosphate pathway which lead to its conversion to hexose phosphate (Glucuronic acid  $\rightarrow$  gulonic acid  $\rightarrow$  L-xylulose  $\rightarrow$  xylitol  $\rightarrow$  D-xylulose  $\rightarrow$  pentose phosphate pathway  $\rightarrow$  hexose phosphate). The glucuronic acid oxidation pathway apparently serves no essential function in man.

4 Strong evidence has been cited to indicate a block in this pathway at some reaction beyond the L-xylulose step in individuals with pentosuria.

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## METABOLISM OF FRUCTOSE

The keto-hexose fructose is widely distributed among plants and is an important source of dietary carbohydrate. It is present as the free monosaccharide or in polysaccharides such as sucrose and less frequently as inulin and raffinose. The open chain form of the hexose is in equilibrium with the corresponding pyranose and furanose forms. In sucrose the fructose in glycosidic linkage is in the furanose form whereas in solutions of the free sugar the pyranose form predominates.

### *Intestinal Absorption*

Fructose polysaccharides are split in the intestine to yield free fructose as the end product of digestion. Fructose as such is not actively transported by the intestinal mucosa as are the other common hexoses glucose and galactose [9]. Some of the fructose is converted to glucose in the intestinal mucosa [10-35]. The remainder that is not further metabolized crosses the mucosa by the process of passive diffusion. The fraction of fructose which is converted to glucose in the passage across the intestinal wall varies considerably from species to species. For example considerably greater conversion occurs in the guinea pig than in the rat [11]. In man it has been estimated that about one sixth of the fructose absorbed is converted to glucose by the intestinal mucosa [12]. This occurs by way of the fructose 1 phosphate pathway discussed in detail below.

### *Plasma Turnover*

Once absorbed fructose is rapidly metabolized. In normal man the half time of disappearance of fructose from the blood following intravenous injection is about 18 min. The concentration of fructose in the blood in the postabsorptive state is normally low ( $2.6 \pm 1.9$  mg per 100 ml) [13]. Following an oral fructose load of 40 to 50 gm in normal individuals the fructose concentration in the blood rises only slightly in 30 to 60 min and rarely above 10 to 20 mg per 100 ml (fructose tolerance test) [3-4]. Less than 2 to 3 per cent of the orally administered fructose appears in the urine.

### *Biochemical Sequences of Fructose Metabolism*

Knowledge of the pathways of fructose metabolism has been obtained from many species. Consequently the following discussion is based upon experiments in different animals. Considerable variation exists from tissue to tissue and the exact role of each remains to be determined. The known reactions are summarized in Fig. 5-1. The numbers used in this discussion refer to this figure.

## Chapter 5

### Fructosuria

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*Stephen M. Krane*

#### DEFINITION

Fructosuria is a rare disorder of metabolism in which there are defective utilization and excessive excretion in the urine of the common hexose fructose. With the exception of the cases described by Froesch et al. [1-30] and that reported by Chambers and Pratt [2], the condition is harmless and asymptomatic. Instances of fructosuria are usually discovered in the course of routine testing of urine for reducing substance which upon further investigation proves to be fructose.

The criteria for the diagnosis of fructosuria may be simply stated [3-5]:

1. The sugar must be identified as fructose, i.e., it is fermentable by yeast, gives a positive Schwanoff reaction, and is levorotatory. Its identity should be confirmed by chromatography [6]. The specimen tested must be freshly voided, since fructose may be formed from glucose in alkaline urine in the presence of some bacteria [7].
2. The subject must have normal liver function, since fructose may be excreted in amounts greater than normal in hepatic failure [7].
3. Usually fructose in the urine is dependent upon its intake in the diet; when the sugar is eliminated from food the fructosuria diminishes.
4. The standard glucose tolerance test result is normal.
5. The condition is not influenced by insulin.

Fructosuria appears in both sexes in approximately equal incidence. It has been reported in all age groups from birth to 89 years [7]. The condition is extremely rare. Marble reported only 4 cases among 29,000 cases of melituria seen at the Joslin Clinic [8], and Lasker estimated that the incidence in the general population is approximately one in 130,000 [7].

In order to consider the metabolic defect in fructosuria it is first necessary to discuss the normal metabolism of fructose.

Fructose 1 phosphate can be phosphorylated to fructose 1,6 diphosphate in the presence of 1 phosphate fructokinase (*Reaction 4*). This enzyme is present in muscle but not in liver [16]. No direct isomerase for the conversion of fructose 1 phosphate to glucose 1 phosphate has been demonstrated [16].

**Reactions 5 and 6** Fructose 1 phosphate can be split by an aldolase to dihydroxyacetone phosphate and glyceraldehyde [20]. Studies of Leuthardt suggest that fructose 1 phosphate aldolase may be a different enzyme from fructose 1,6 diphosphate aldolase [21]. Hers on the other hand has suggested that a single aldolase catalyzes both *Reactions 5 and 6* but with different substrate affinities (the  $K_m$  for fructose 1 phosphate is 100 times that for fructose 1,6 diphosphate) [20].

**Reactions 7 to 10** The glyceraldehyde formed by splitting fructose 1 phosphate is phosphorylated in the presence of ATP by a triosekinase to glyceraldehyde 3 phosphate (*Reaction 7*) [20]. This can then condense with dihydroxyacetone phosphate in the presence of aldolase to form hexose diphosphate [20]. In liver and muscle another enzyme has been identified (*Reaction 8*) fructose 1,6 diphosphatase which in the presence of magnesium ion splits the phosphate specifically from carbon 1 of fructose 1,6 diphosphate to yield fructose 6 phosphate and inorganic phosphate [20]. The fructose 6-phosphate can be converted to glucose 6 phosphate (*Reaction 9*) in the presence of phosphohexose isomerase and with glucose 6-phosphatase (*Reaction 10*) in liver free glucose may be formed [16, 20]. Thus a series of reactions has been demonstrated for the conversion of fructose to glucose in the liver.

There is another series of reactions for the interconversion of fructose and glucose. In the seminal vesicles glucose is reduced to sorbitol by TPNH. The sorbitol in turn is oxidized to fructose by DPN. These reactions account for the high concentration of fructose in the semen [22, 23].

### Utilization of Fructose

In man 40 to 50 per cent of the fructose is removed by the liver and plasmatic tissues during a constant infusion [12, 13, 24]. Other estimates of total metabolism of fructose by the liver are as high as 80 per cent [3]. Fructose freely enters hepatic cells [20]. The mechanism of utilization of fructose in the presence of glucose by hepatic cells has been outlined above. There is still considerable uncertainty concerning the extent of metabolism of fructose by muscle. Although utilization of fructose has been demonstrated in isolated muscle and by measurement of peripheral arteriovenous differences in the presence of glucose this is small compared to glucose utilization [24]. In the isolated diaphragm conversion of fructose to glycogen when equal concentrations of glucose and fructose are present is only one-sixth that of glucose and conversion to carbon dioxide only one-third that of glucose [24]. In the excised nephrec

**Reaction 1** Fructose can be phosphorylated at position 6 in the presence of  $\text{ATP}$ , magnesium ion, and hexokinase. The enzyme which has been most carefully studied is that located in the particulate fractions of brain. It catalyzes the phosphorylation of fructose, but the affinity of the enzyme for fructose is considerably lower than for glucose [14]. Therefore, at equimolar concentrations of glucose and fructose, phosphorylation of fructose by this enzyme is completely inhibited [15]. If the relative affinities of other tissue hexokinases are similar to that of brain hexokinase, then this enzyme has little importance for phosphorylation to fructose 6-phosphate because of the unfavorable ratios of glucose to fructose.

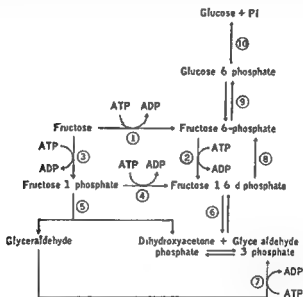


Fig. 5-1 The pathways of fructose metabolism. The reaction number refers to the text.

**Reaction 2** Fructose 6-phosphate is further phosphorylated to fructose 1,6-diphosphate in the presence of magnesium ions,  $\text{ATP}$ , and 6-phosphofructokinase. The activity of this system is low in liver but high in muscle [16]. Under conditions of high fructose concentration, muscle phosphofructokinase can phosphorylate fructose in the 1 position because of its lack of specificity [17]. However, there is probably little physiologic significance to the latter reaction.

**Reaction 3** In liver and possibly also in muscle, another enzyme, fructokinase, catalyzes the phosphorylation of fructose in the 1 position. It has an absolute requirement for potassium and magnesium ions and is inhibited noncompetitively by ADP [16, 18, 19]. In contrast to the less specific hexokinase, glucose is not a substrate for fructokinase and does not competitively inhibit the phosphorylation of fructose by the latter enzyme.

Sachs Sternfeld and Kraus reviewed 50 cases of fructosuria reported until 1942 and added 2 cases of their own [4]. Several cases have been published since then [3, 8, 30, 31]. The following discussion represents a summary of the material in the reports.

### *Abnormalities in Fructose Utilization*

In the majority of patients no more than traces of fructose are found in the urine and blood in the fasting state. An exception is the patient reported by Lenzner who had a fasting blood fructose of 41 mg per 100 ml and 0.4 gm fructose in a 2 hr urine collection after 15 hr of fasting [31]. However following an oral fructose load or the ingestion of fructose either as the mono- or polysaccharide the hexose appears in the urine. Within 3 to 4 hr following fructose ingestion 10 to 20 per cent of the sugar appears in the urine in contrast to normal subjects who excrete only 1 to 2 per cent under similar circumstances. On an average diet the fructosuric subject rarely excretes urine with fructose concentrations greater than 2 to 3 per cent but values fluctuate widely depending upon intake [3, 4, 8].

The patient with fructosuria shows a significant rise in blood fructose following fructose feeding. Whereas normal persons rarely reach concentrations higher than 10 to 20 mg per 100 ml after ingesting 50 gm fructose fructosuric patients often show rises as high as 65 mg per 100 ml 1 to 2 hr later.<sup>1</sup> In several fructose tolerance tests in fructosuric subjects the blood glucose has fallen at the time when fructose concentrations have reached peak values [4]. When one subject was given 50 gm each of glucose and fructose simultaneously the urinary excretion of fructose was greater than that with fructose alone. This was interpreted as indicating that glucose competitively inhibits the utilization of fructose [8].

Sachs Sternfeld and Kraus in their review [4] cited earlier observations that in fructosuria the expected rise in respiratory quotient (R.Q.) and in blood lactic acid following fructose ingestion did not occur [32, 33]. Heeres and Vos observed that their patient with fructosuria had an increase in R.Q. from 0.72 only to 0.77 following 50 gm fructose orally whereas a normal person treated similarly had a rise in R.Q. from 0.71 to 0.90 [33]. In the two cases of fructosuria reported by Sachs et al. the increases in blood lactic acid were 2 and 1 mg per 100 ml 1 hr after fructose ingestion compared with increases of 13 and 12 mg per 100 ml in two controls [4]. In their fructosuric persons serum inorganic phosphate did

The levels of blood fructose reported by Lenzner [31] in both the fructosuric and control subjects are higher than any others reported. On the basis of the blood values the fructose tolerance test result of his control subject would be considered markedly abnormal and compatible with the diagnosis of fructosuria. Although in the fructose method used by him glucose was stated not to interfere details of recovery experiments are not given.

tomized rabbit the rate of formation of  $C^{14}O_2$  from fructose  $C^{14}$  is only 5 per cent of that from glucose  $C^{14}$  [26]. Recent experiments of Hers using fructose  $C^{14}$  have shown that most of the fructose converted to muscle glycogen is derived from the glucose formed from fructose in the liver [27]. The studies of Sols have also suggested that fructose utilization (fructose 1 phosphate formation) by muscle extracts is a side activity of phosphofructokinase [17]. There are few data concerning fructose utilization by other tissues. Erythrocytes for example utilize fructose only in the absence of glucose [1]. Lactic acid production by leukocytes in equimolar concentrations of fructose and glucose is one-quarter that with fructose alone [28].

The available evidence thus suggests that the important pathway for fructose utilization is through the liver rather than through muscle in contrast to glucose. Infusions of fructose to normal man produce a considerably greater rise in lactic and pyruvic acids and a greater fall of inorganic phosphate in the serum than similar infusions of glucose [18]. The hepatic uptake and conversion of fructose to lactate, carbon dioxide and glycogen exceeds that of glucose [24]. However studies of Smith, Pittenger and Seligson indicate that the rates of utilization of infused fructose and glucose are approximately equal [18]. The authors therefore have suggested that the differences in the ability of the two hexoses to produce a rise in lactate and pyruvate is accounted for by the entrance of fructose into the Embden Meyerhof scheme at a different level than glucose. The first product of fructose metabolism, fructose 1 phosphate, is immediately metabolized to 3 carbon fragments as was discussed above.

The renal threshold for fructose is apparently quite low; urine to plasma ratios of fructose of less than 1.0 are found only at plasma concentrations below 15 mg per 100 ml [29]. At fructose levels higher than this some sugar will appear in the urine. Smith has summarized the evidence which suggests active reabsorption of fructose by the renal tubules from the glomerular filtrate [29].

Several studies have shown that fructose utilization is normal in diabetic man [12, 13]. The rate of fructose disappearance following intravenous infusion is only slightly prolonged in diabetic human subjects and changes in blood lactic, pyruvic,  $\alpha$ -ketoglutaric acids and inorganic phosphate are of the same magnitude as in normal persons [13]. Insulin fails to influence fructose utilization [8, 13].

### THE METABOLIC ABNORMALITY IN BENIGN FRUCTOSURIA

In the light of the previous discussion of fructose metabolism the abnormalities in fructosuria may be considered together with hypotheses regarding the mechanism of the defect.

The child gradually learned to avoid sweets. Aside from slight enlargement of the liver, small stature and decreased intelligence, no other abnormalities were found. Glucose and galactose tolerance test results were normal. There was no hypersensitivity to fructose by skin testing. Paper chromatography of the urine revealed some increase in amino acids. The child was given 50 gm fructose per square meter of body surface and fructose and glucose (the latter by glucose oxidase method) were determined in blood and urine. Within 40 min the child became pale, cyanotic, irritable and began to sweat excessively. The blood pressure rose from 115/65 before the test to 135/85 in 4 hr. At 40 min the blood glucose had fallen from a fasting concentration of 65 mg per 100 ml to 35 mg per 100 ml and the blood fructose had risen from trace amounts to over 80 mg per 100 ml. Ninety minutes after the test was begun the blood fructose reached a peak of 138 mg per 100 ml and the blood glucose was 8 mg per 100 ml. After 4 hr there was still profound hypoglycemia and hyperfructosemia. The blood glucose did not begin to rise until after 6 hr, when the blood fructose had fallen to below 30 mg per 100 ml and slight clinical improvement began. The patient was still nauseated and exhausted on the following day. Two days after the test, mild icterus and albuminuria developed. During the study only 10 per cent of the fructose was excreted in the urine.

A similar clinical picture was observed in the 2½ year old brother of the patient reported above. Mention is also made of two adult relatives of these children who were strong and healthy in appearance but who had avoided fruits and sugar because during early life these had produced episodes of nausea, vomiting and somnolence. A smaller fructose load was given to one of these. The blood fructose rose from a fasting level of 54 mg per 100 ml to a peak of 86 mg per 100 ml. The blood glucose dropped concomitantly to 20 mg per 100 ml and the drop was accompanied by hunger, sweating and palpitations. Fructose tolerance test results in the parents of the two children described above were normal. Peak values of fructose in the blood were only 17 mg per 100 ml and only traces of fructose appeared in the urine during the test. Of six parents of the four patients described, four could be traced to common ancestors in the eighteenth century.

The authors explain the abnormality in these patients as follows. They note that in previous studies of fructosuria, mild decreases in blood glucose concentrations have been seen in the course of fructose tolerance tests. Although it had been observed that in cross-circulation experiments in dogs [34] there was no stimulation of insulin production with fructose, the authors suggest that this does occur in man. In normal man the conversion of fructose to glucose in the liver is prompt and serves to correct the hypoglycemia to some extent. In their patients this fructose-to-glucose conversion is blocked.



not fall as it normally does following fructose ingestion. A similar failure to demonstrate a rise in lactic acid in fructosuria was noted by Levine and Labenhaut [3].

It can be seen that there is considerable evidence that the utilization of fructose is decreased in fructuric subjects. In these individuals following the ingestion of fructose there is an excessive rise in blood fructose and a failure both in production of lactic acid and  $\text{CO}_2$  (failure of R.Q. to increase) and in consumption of inorganic phosphate. In addition there is a decrease in the conversion of fructose to glucose as may be inferred from the results of fructose tolerance tests in fructuric subjects; there is a slight fall in glucose concentration in the blood in contrast to a slight rise in normal persons. It has often been stressed that recovery of fructose in the urine of fructuric subjects is rarely greater than 20 per cent of the load. It is then assumed that 80 per cent of the sugar is "utilized" [3]. "Disappearance" can be equated with utilization only when products of metabolism can be demonstrated as has been pointed out by Smith et al. [19]. In fructosuria no increase in fructose metabolites (such as lactate) occurs after fructose ingestion. What happens to the remaining 80 per cent of the fructose load is unknown.

#### *Suggested Enzymatic Defects*

At the present time the evidence favors the interpretation that the defect in fructosuria is a deficiency of hepatic fructokinase (Reaction 3) [1]. An alternative explanation places the defect not at fructokinase but in the utilization of fructose 1 phosphate (fructose 1 phosphate-aldolase Reaction 5). As mentioned previously, the enzyme for the conversion of fructose 1 phosphate to fructose 1,6 diphosphate is not present in the liver. A defect in the utilization of fructose 1 phosphate in liver could account for the disappearance of fructose not excreted in the urine and not appearing as lactate, glucose, or  $\text{CO}_2$ . Fructose 1 phosphate would then be expected to accumulate in the cells. It must be emphasized that the evidence is inferential and indirect and will probably remain so until there are opportunities for directly measuring the activities of the enzymes involved in fructose metabolism in affected persons.

### FRUCTOSURIA WITH HYPOGLUCOSEMIA

Although usually no clinical disturbance is associated with fructosuria a recent report by Froesch et al. documents striking fructose intolerance in several members of a Swiss family [1]. Their first patient was a 6½ year old girl who was retarded in growth and intellectual development. The child's mother had recognized since age 8 months that frequent attacks of vomiting, sweating, somnolence, and tremor were associated with the ingestion of cane sugar and fruits. Pure glucose was well tolerated

2 The defect is not found to occur in any of the parents or offspring of the affected subjects

3 In 6 of 7 families where the facts are known the parents of the patients are related in some manner

Froesch et al conclude that the inheritance in their cases of fructosuria with hypoglycosemia is an autosomal recessive [1] The same criteria proposed by Lasker would be fulfilled in this instance i.e. occurrence in siblings nonoccurrence in parents and evidence of consanguinity in the parents of the affected subjects

## TREATMENT

No treatment is indicated in the benign form of fructosuria since there are no symptoms and no evidence that there are any deleterious effects from fructose ingestion The only clinical hazard is confusion of the condition with diabetes mellitus The development of specific and simple tests for urine glucose involving glucose oxidase has minimized this danger<sup>2</sup> For those subjects with fructosuria and hypoglycosemia absolute restriction of fructose in all forms in the diet is mandatory if cerebral damage is to be avoided

## SUMMARY

1 Fructosuria is usually a benign asymptomatic metabolic anomaly Following ingestion of fructose affected persons do not metabolize the sugar normally There result a high concentration of fructose in the blood and failure of the blood lactate or respiratory quotient to rise in contrast to what is observed in normal persons

2 There is no evidence to suggest a renal defect but the sugar is excreted in the urine when the renal threshold is surpassed Although definitive studies have not been reported it is possible that a deficiency in fructokinase of liver and other tissues is responsible for the defect

3 Another group of patients has been described who have profound depression in glucose concentration in the blood associated with hyperfructosemia and fructosuria following the ingestion of fructose It has been postulated that in these subjects the defect in fructose utilization involves a deficiency of fructose 1 phosphate aldolase or glyceraldehyde kinase

4 Both forms of fructosuria are probably inherited as Mendelian recessive traits

<sup>2</sup>On the other hand if it is a high glucose oxidase for sugar the urine are with a 1 pt 1 for routine screening purposes and 1 pt specific tests for reducing substances are discarded then interesting abnormalities such as fructosuria will go undetected

In a more recent report Froesch et al [36] have made several additional observations on these interesting subjects. They found that when a small dose of fructose (3 gm per m<sup>2</sup> of body surface) was given intravenously to the younger brother of the patient mentioned above a fat exaggerated and prolonged fall in serum inorganic phosphorus occurred in contrast to what occurred in a normal slightly older subject. On the basis of this observation they postulated that fructose was normally phosphorylated to fructose 1 phosphate and that this ester accumulated within the cell because of a primary deficiency of fructose 1 phosphate aldolase. Unfortunately the authors were unable to obtain liver biopsies to prove this and their conclusions are therefore inferential. In one experiment with a rat liver homogenate the authors observed 60 to 70 per cent inhibition of fructokinase with fructose 1 phosphate present at a concentration of  $5 \times 10^{-3} M$ . Inhibition of fructokinase by fructose 1 phosphate was not previously noted [16]. Using crystalline aldolase they observed competitive inhibition of this enzyme by fructose 1 phosphate and calculated an inhibition constant to be about  $10^{-3} M (K_i)$ . On the basis of these observations they postulated that accumulation of fructose 1 phosphate could inhibit fructose utilization as well as glucose formation via the aldolase reaction.

In attempting to explain the difference between their cases and the usual patients with benign fructosuria, Froesch et al have speculated that in their cases the defect is in fructose 1 phosphate aldolase (Reaction 5) or glyceraldehyde kinase (Reaction 7). They make the valid observation that it is improbable that their cases are homozygous and those with benign fructosuria are heterozygous for the same defective gene since the parents of their cases had normal fructose tolerance to the tests. Whether or not this is a qualitatively different form of fructosuria will be decided only by definitive localization of the defect.

Chambers and Pratt have reported the case of a 24 year-old woman who had recurrent vomiting following ingestion of fruit or cane sugar [3]. The peak blood fructose level was 17 mg per cent at 60 min after eating 50 gm fructose. No blood glucose data were reported.

## GENETICS

The most careful study of the mode of inheritance of fructosuria is that of Lasker [7]. She reviewed the literature and her own case and concluded that the disorder is inherited as a Mendelian recessive trait. Her arguments are as follows:

1. Of the 19 families on whom adequate data are available, 10 show the defect in more than one sibling. In 15 families where there is information on the total size of the sibship, 40 brothers and sisters of the original cases were tested for fructosuria and 7 were found to be positive.

2 The defect is not found to occur in any of the parents or offspring of the affected subjects

3 In 6 of 7 families where the facts are known the parents of the patients are related in some manner

Froesch et al conclude that the inheritance in their cases of fructosuria with hypoglycemia is an autosomal recessive (1) The same criteria proposed by Lasker would be fulfilled in this instance i.e. occurrence in siblings nonoccurrence in parents and evidence of consanguinity in the parents of the affected subjects

## TREATMENT

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## SUMMARY

1 Fructosuria is usually a benign asymptomatic metabolic anomaly. Following ingestion of fructose affected persons do not metabolize the sugar normally. There results a high concentration of fructose in the blood and failure of the blood lactate or respiratory quotient to rise in contrast to what is observed in normal persons.

2 There is no evidence to suggest a renal defect but the sugar is excreted in the urine when the renal threshold is surpassed. Although definitive studies have not been reported it is possible that a deficiency in fructokinase of liver and other tissues is responsible for the defect.

3 Another group of patients has been described who have profound depression in glucose concentration in the blood associated with hyperfructosemia and fructosuria following the ingestion of fructose. It has been postulated that in these subjects the defect in fructose utilization involves a deficiency of fructose 1 phosphate aldolase or glyceraldehyde kinase.

4 Both forms of fructosuria are probably inherited as Mendelian recessive traits.

<sup>1</sup>On the whole, rapid tests using glucose oxidase for sugar in the urine are suitable for routine screening purposes and less specific tests for reducing substances are discarded. Then interesting abnormalities such as fructosuria will go undetected.

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## Chapter 6

### Glycogen Deposition Diseases\*

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*Richard A. Fuld*

Glycogen deposition diseases occupy a noteworthy place in a treatise on the biochemical aspects of hereditary diseases for the demonstration by Gerty T. Cori in 1952 [1] that von Gierke's disease is due to the loss of activity of a single known tissue enzyme marked the first clear delineation of an inborn error of metabolism in enzymatic terms and served as a prototype for future work in this group of diseases. The contributions of specific enzymologic studies of tissues from patients with genetically determined metabolic aberrations in which Dr. Cori led the way, have already been voluminous and significant. The demonstration of the practicability of a new conceptual approach to the study of the diseases was among Dr. Cori's greatest contributions and will continue to adorn the memory of this great scientist and thinker. To those who knew her and had the experience of working with her, the memory of her imagination, energy, and power of intellect will always serve as a source of inspiration [2].

As summarized by Dr. Cori in her last publication on the subject [3], enzymatic investigations have made it possible to classify cases of glycogen deposition diseases according to a specific enzyme defect and the resulting alterations of structure of the accumulated tissue glycogen. Such a classification is based on an ever-expanding knowledge of the successive steps of glycogen formation and breakdown. A review of these facts follows.

#### METABOLISM OF GLYCOGEN

Glycogen, a glucosyl polymer polysaccharide, has a role as a reserve material in animal carbohydrate metabolism analogous to the role of

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starch in plant life. Apparently every tissue of the body can form glycogen from glucose. glycogen is most abundant in the liver and muscle. Under normal conditions in man there are 300 gm of muscle glycogen and 150 to 200 gm of liver glycogen. Approximately 25 per cent of the usual 150 to 350 gm of carbohydrate in the diet is stored as glycogen. Although this storage polymer of glucose is available in other tissues as a source of hexose phosphate for their intrinsic metabolic needs, only the liver can release significant quantities of free glucose into the blood for transport to other tissues. In the rat the biologic half life of liver glycogen is 1 day whereas it is 3 to 4 days elsewhere in the body. This reflects the primary role of the liver in maintaining normoglycemia and carbohydrate homeostasis. The ability of hepatic glycogen to supply glucose to the blood stream is almost entirely dependent upon the presence of a relatively specific hepatic glucose 6-phosphatase. An identical or very similar specific glucose 6-phosphatase is found in the kidneys and islet cells of the pancreas [4] (and perhaps in the mucosa of the ileum) but it seems unlikely that these organs contribute much dephosphorylated glucose to the blood stream. The presence of this enzyme in the proximal convoluted tubules of the kidney [5] suggests that its role in this organ concerns tubular reabsorption of glucose from the glomerular filtrate (cf Chap 38).

### *Structure of Glycogen*

This polysaccharide is a high molecular weight polydisperse polymer (2.5 to 4.5 million average molecular weight) consisting of only one building block,  $\alpha$ -D-glucose [6]. Many molecules contain more than 10,000 glucosyl units. The prevalent link between the glucose residues is in the  $\alpha$  configuration through C atoms 1 and 4. In addition, there are a lesser number of  $\alpha$ 1,6-glucosidic bonds which constitute branch points and impart an arborized structure to the molecule. The percentage of glucosyl moieties involved in formation of the  $\alpha$ 1,6 branch points varies between 6 and 11 per cent of the total in glycogens from different organs or from different species. A free reducing group exists only at one terminus of the molecule. The uninterrupted succession of 1,4-linked glucosyl components terminates after successive branching and unbranching at 1,6 linkage points with an end devoid of reducing capacity. In this ramified structure the external chains which are situated beyond the last 1,6 branch point are much longer than the internal chain located between two points of ramification. The outer chains consist of from 7 to 10 glycosyl units in man and constitute approximately 40 per cent of the mass of the molecule, while the average length of the inner chains is four glucose residues [7]. The resultant structure is more highly branched than amylopectin, the analogously branched component of starches, and consequently both inner and outer chains of glycogen are shorter. This is correlated with the

much higher solubility of glycogen than of amylopectin in water and also accounts for a Bordeaux color reaction with iodine rather than the purple one obtained with starch. There may be other differences between these two types of polysaccharides, nevertheless it seems justified and useful to regard them as belonging to a family of closely related molecules [3] serving similar physiological roles (Fig. 6-1).

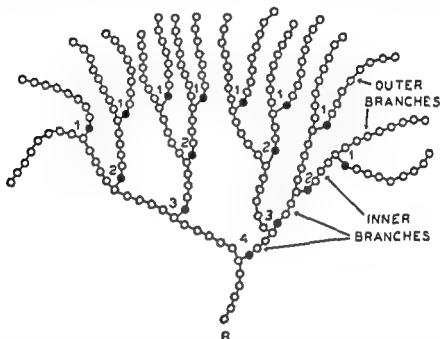


Fig. 6-1 Model of a segment of a glycogen molecule. There are 63 glucose residues (molecular weight 33,858). Open circle = glucose residues in  $\alpha$  1-4 linkage. 1 = reducing end group. There are four tiers of branch points (glycogen has at least seven). Inner branches are terminated by branch points in adjacent tier; outer branches by a branch point and by the nonreducing terminal glucose residue (end group). (By permission of C. T. Cori [3].)

### Synthesis and Degradation of Glycogen

In the various classified types of abnormal glycogen deposition syndromes the lesions involving the glycogen molecule are both quantitative and qualitative. Normally liver glycogen content in man varies from 0 to 5 per cent depending on circumstances. Muscle glycogen is only rarely as high as 1 per cent of the wet weight of the tissue. Excessive deposition of glycogen to the extent of 15 per cent of wet weight of liver (approximately 50 per cent of the dry weight) and up to 12 per cent of the wet weight in the muscle has been found [8]. The classification and characterization of the qualitative disturbances of the structure of the glycogen molecule are best described as logical consequences of specific



enzyme defects in pathways traversed by glucose in the synthesis of glycogen and in the breakdown of glycogen to yield again free glucose. These reactions bear close scrutiny inasmuch as they influence the availability of glycogen and are central in carbohydrate metabolism. Disorders of glycogen synthesis and limitations of the availability of glucose from glycogen can understandably exert far reaching effects demonstrable chiefly as hypoglycemia, impairment of synthetic processes and of emergency release of energy and limitation of growth. The schema of Fig. 6-2A and B above summarizes current knowledge of the steps in the liver (Fig. 6-2).

Presentation of glycogenesis and glycogenolysis as separate pathways enhances the clarity of the discussion of the individual enzymes. The recent demonstration of UDPG: glycogen transglucosylase by Leloir [9] has provided a mechanism for a division of the two processes beyond the stage of glucose 1 phosphate formation. As pointed out by Robbins et al. [10] and Leloir et al. [11] in their more elaborate characterization of this enzyme such a partition would resolve the long standing paradox between the classical concept of reversible interconversion of glycogen and glucose 1 phosphate and the findings of Sutherland and coworkers [12-14] and of Cori [15] that activation of phosphorylase *in vivo* invariably leads to glycogen breakdown and never to glycogen synthesis. It is tempting to add as further evidence for partition the demonstrated ability of human muscle lacking phosphorylase activity to synthesize glycogen. As yet direct evidence excluding phosphorylase from participation in glycogen synthesis in the intact normal animal has not been presented. Nevertheless it seems likely that such evidence will be forthcoming. Thus it has been stressed by Stetten and others that the normal tissue concentrations of inorganic phosphate are relatively high in relation to determined concentrations of glucose 1 phosphate. This relationship would keep the phosphorylation reaction well over on the breakdown side of its equilibrium (see below).

### GLYCOGENOLYTIC ENZYMES

#### *Phosphorylase*

This enzyme *in vitro* is specific for the phosphorylytic breaking of the prevalent  $\alpha$  1-4 linkage in glycogen (Fig. 6-3).

*In vitro* the reaction is easily reversible with an equilibrium constant [16]

$$\frac{(\text{Phosphate})}{[\text{Glucose 1 phosphate}]} = 2.45 \text{ at pH } 7.33$$

Rabbit muscle phosphorylase as crystallized by Green and Cori [17] and called phosphorylase  $\alpha$  has more than 60 per cent of its activity without

Uridine diphosphoglucose

addition of nucleotides but requires the addition of adenosine 5 mono-phosphate for maximal activity *in vitro*. Phosphorylase  $\alpha$  is apparently a tetramer which contains 4 moles of pyridoxal phosphate per mole [18]. It can be converted by the hydrolytic PR or phosphorylase rupturing enzyme abundantly present in muscle tissue into a dimer with

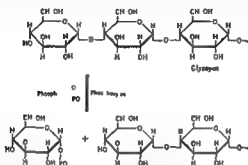


Fig 6-3 The active phosphorylase reaction *in vitro* (By permission of C T Cors [3])

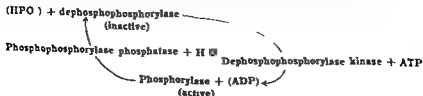
an absolute requirement for AMP for activity [19]. The inactivating cleavage reaction has been summarized as [20]



Recondensation of two dimers into an active  $\alpha$  tetramer requires a specific phosphorylase  $\beta$  kinase and ATP and proceeds as follows [21]



Liver possesses a phosphorylase which is immunologically distinct and biochemically different from the phosphorylase of muscle [22] but which also exists in an active and inactive form. In the case of hepatic phosphorylase in contrast to the situation with muscle phosphorylase deactivation of the active form by a specific enzyme does not involve a cleavage. The active and inactive forms have the same molecular weight. Sutherland and Wolcott [23] purified the active form and Sutherland and coworkers [24] described a system of phosphorylation and dephosphorylation of hepatic phosphorylase in which the amount of active phosphorylated enzyme present at any instant is the resultant of the continuous dynamic actions of the activating and deactivating enzyme. The situation can be described graphically as follows:



The activity of the phosphorylated enzyme can be increased 15 to 40 per cent *in vitro* by the addition of AMP. Glycogenolytic activity of liver tissue at any given instant seems to be directly proportional to its content of active phosphorylated phosphorylase [25-27] which is in keeping with the demonstration that the phosphorylase step is rate limiting in glycogenolysis [28]. The relative amount of active phosphorylase, as will be discussed in detail later, can be greatly enhanced by the actions of epinephrine and glucagon invariably with a concomitant increase in breakdown of glycogen. This is a further indication of the nonintervention of phosphorylation in glycogenesis and supports the notion that synthesis occurs primarily via UDPG and the Leloir enzyme. A deficiency of muscle phosphorylase also to be described later results in a form of excessive glycogen deposition in muscles, a circumstance which would be hard to understand if the enzyme were required in both formation and depolymerization of the polysaccharide. Thus pending direct evidence on the point it seems proper to assign to phosphorylase as its chief if not sole physiologic role the phosphorylation of glycogen and to postulate that synthesis occurs chiefly via the Leloir enzyme. If phosphorylase does participate in synthesis to any degree then some more subtle circumstances of physical partition within the cells or more complicated influence for intermittent reversal of the direction of action than the phosphate: glucose 1-phosphate ratio must exist.

### *Amylo-1-6-glucosidase*

In its action during glycogenolysis phosphorylase successively attacks the most distal  $\alpha$  1-4 glucosidic bonds of the outer branches of the treelike glycogen molecule. In the presence of adequate concentrations of inorganic phosphate the successive phosphorolytic cleavages continue until the outermost tiers of  $\alpha$  1-6 glucosidically bound glucosyl moieties are approached. Since crystalline or purified phosphorylase cannot attack this linkage action stops and the results are an accumulation of glucose 1-phosphate, stoichiometric consumption of inorganic phosphate and a partially depolymerized glycogen tree exhaustively pruned of its outer branches. The partially depolymerized but not yet deramified polysaccharide which results from exhaustive phosphorylase action has been termed the *first limit dextran* (phosphorylase) or *LD<sub>1</sub>* (phosphorylase) (Fig. 6-4).

*LD<sub>1</sub>* (phosphorylase) is not identical with the one produced by the plant enzyme  $\beta$  amylase. The latter can approach the branch points more closely while splitting off the disaccharide maltose. The *LD<sub>1</sub>* ( $\beta$  amylase) is a smaller molecule than *LD<sub>1</sub>* (phosphorylase) as can be appreciated by study of Fig. 6-4.

Another enzyme, amylo-1-6-glucosidase present in muscle and presumably liver catalyzes the hydrolytic cleavage of the 1-6 linked glucose

residues [29]. This enzyme appropriately called 'debrancher', acts only on the LD<sub>1</sub>, LD<sub>2</sub>, LD<sub>3</sub> etc (phosphorylase) in which the glucosyl residues in 1-6 linkage are no longer covered by additional glucose residues. It has no significant activity on undigested glycogen, amylopectin or on LD<sub>1</sub> ( $\beta$ -amylase). The debrancher reaction is from physiologic considerations irreversible. In splitting the branch point with the introduction of H<sub>2</sub>O it yields free glucose rather than glucose-1-phosphate.

Limit dextrin (phosphorylase)  $\longrightarrow$  dextranized glycogen + glucose

Although only 8 per cent of the glycogen appears as free glucose via this mechanism when glycogen is enzymatically degraded *in vitro* the

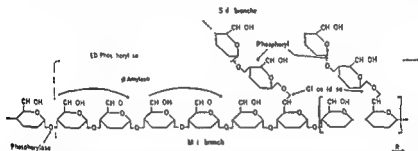


Fig 6-4 Structural model of portion of a branched polysaccharide showing the sites of enzymatic action. LD corresponds to the limit dextrin formed by exhaustive action of phosphorylase. I refers to the reducing end (B, permission of G. I. Cors [3]).

recognition of this potential source of free glucose is important in understanding the occurrence of hyperglycemia following administration of glucagon and epinephrine in certain of the glycogen deposition diseases.

The purification of this enzyme and its removal as a contaminant in the preparation of phosphorylase by repeated crystallizations of the latter have provided investigational tools for determination of the structure of various glycogens and an elegant method of biochemical diagnosis [30]. By the successive actions of crystalline phosphorylase and purified debrancher in a tierwise fashion on purified glycogen, the length of the outer chains, number of branch points in any given tier, length of inner chains, and number of tiers can be deduced. As will be seen, this method of characterization of qualitatively abnormal glycogens led to the postulation of specific enzyme lesions. Eventually direct analysis of the enzyme in question proved that the hypothesis based on enzymatic structure analysis was correct [31]. Thus, by the joint action of phosphorylase and amylo-1,6-glucosidase, a molecule of glycogen can be almost completely degraded to glucose-1-phosphate and glucose, leaving as a residue an oligosaccharide whose molecular weight and structure

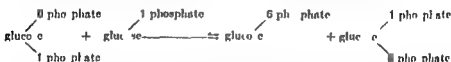


are not well known



### *Phosphoglucomutase*

Abnormalities of this enzyme have not been described as a cause of biochemical pathologic change. Much information has been obtained concerning its mode of action. It has been surmised that absence or deficiency of this enzyme would be lethal [8] because of its bottleneck position in glycogen metabolism. The enzyme mediates the interconversion of glucose 1 phosphate and glucose 6-phosphate and requires glucose 1,6-diphosphate as an obligate cofactor *in vitro* [32]. The reaction is thought to proceed as follows:



At equilibrium the mixture contains 95 per cent glucose 6-phosphate and 5 per cent glucose 1 phosphate [33], which makes it difficult to see how glycogen synthesis is affected via this enzyme if the initial source material is glucose 6-phosphate nevertheless no specific evidence is available which casts doubt on its intervention in the formation of glycogen as well as in its degradation. Interference with phosphoglucomutase activity by galactose 1-phosphate has been demonstrated [34-35] and postulated as an important cause for the problems seen in galactosemic children fed on galactose or lactose (Chap. 7).

### *Glucose 6-phosphatase*

This microsomal enzyme accomplishes the hydrolytic cleavage of phosphate from glucose 6-phosphate liberating free glucose and inorganic phosphate [36-37]. It is normally present in abundance only in the liver and kidneys [38], a circumstance which explains the anatomic distribution of excessive glycogen deposition in these organs when lesions of this enzyme exist. Liver has other phosphatases which are capable of attacking glucose 6-phosphate but under physiologic conditions the specific glucose 6-phosphatase dephosphorylates at least ten times the amount of glucose 6-phosphate as the nonspecific enzymes [8]. The nonspecific predominantly acid phosphatases of liver have very little activity in the physiologic range of pH 6.2 to 6.8 in which the optimum for the specific microsomal enzyme lies [1]. Further glucose 6-phosphatase has a preferential affinity for glucose 6-phosphate (Michaelis constant  $5 \times 10^{-3} M$ ) [39] and there is little splitting of other hexose 6-phosphates when they are tested with this enzyme in the same concentration [40].

The enzyme attacks phosphate esters of primary alcohols with considerable avidity [39]

It is essential to the understanding of the manifestations and responses of subjects bearing a deficiency of this enzyme to recognize its influential and critical position in the regulation of the blood sugar. In the fasting state glucose 6-phosphate derived either from glycogenolysis or from the formation of glucose from other than carbohydrate sources (gluconeogenesis) requires the intercession of this enzyme before it can leave the liver and enter the blood as free glucose. With the exception of the small amount of glucose formed during deramification by amylo-1,6-glucosidase all blood sugar of endogenous origin comes via glucose 6-phosphatase.

An interesting and probably physiologically important property of this enzyme is that its level of assayable activity is decidedly influenced by hormones, drugs, and circumstances which are attended by shifts in the direction of liver glucose metabolism [41]. The assayable activity rises in diabetes, starvation, or whenever the liver is provoked to increase its output of free glucose. Conversely, circumstances such as hyperalimentation or administration of insulin or sulfonylurea [42] are attended by a drop in activity of this enzyme. These changes in enzyme activity occur only *in vivo* and require some time and persistent change in conditions to become manifest [43]. Increases and decreases in activity seem to parallel the presumed changes in intracellular glucose 6-phosphate concentration or production, rising in response to the persistence of increased levels of the substrate and falling following sustained declines in the availability of the substrate. It is not clear whether these adaptive changes are brought about by increased enzyme synthesis such as have been described for so-called adaptive enzymes, or whether shifts in levels of activity represent alterations in a ratio of active and inactive forms of a relatively fixed number of enzyme molecules, such as seems to be the case with liver phosphorylase. Treatment of microsomal particles rich in glucose 6-phosphatase activity with Triton and other detergents has been shown to increase markedly the activity *in vitro* [42], suggesting a possible mechanism by which shifts between an active and an altered active form might be effected *in vivo*. It is conceivable that the clinical examples of deficiencies of this enzyme activity, although they are almost absolute, represent a genetically determined defect in an activating-deactivating system rather than an absolute inability to produce the enzyme molecule. The question is more than academic since the possibility of a therapeutic approach to the deficient activity syndrome might be easier if the problem is to find a method of activating an existing enzyme rather than to induce *de novo* production of enzyme molecules or to induce a metabolic bypass. Substitution therapy by supplying glucose 6-phosphatase from exogenous sources seems hardly possible on immunologic grounds and the improbability

of the enzymes finding its proper place inside the proper cell has been pointed out by Cori [3]

### GLYCOGENIC ENZYMES

Only a single instance of a disordered glycogen synthesis marked by excessive glycogen deposition and an abnormally structured polysaccharide has come under study [31]. Despite this apparent paucity of examples of disordered glycogen synthesis recent discoveries concerning this obviously important side of the glycogen cycle make it profitable to discuss the steps involved in some detail.

#### *Hexokinase*

The concept that phosphorylation occurs during entry of sugars into cells has been under assault in recent years as a result of studies on the mechanisms of insulin action [45] and intestinal absorption [46]. The liver cell has long been recognized as relatively highly permeable to free glucose and it is not difficult to demonstrate definite concentrations of this hexose in the intracellular water of hepatic cells [47]. In order to enter the steps of glycogen formation or carbohydrate metabolism glucose and other sugars require phosphorylation by ATP and a "kinase." The properties of muscle hexokinase [48] and of brain hexokinase [49] which mediate the transfer of the terminal phosphate from ATP to C6 of glucose have been extensively studied. The liver is more difficult to study because of the apparent lability of the enzymes, interferences by ATPases and the difficulties in obtaining preparations of phosphoglucomutase and other contaminating enzymes. A specific glucokinase for liver which phosphorylates in the 6 position has been postulated [50]. For fructose and galactose [50-51] specific kinases mediate phosphorylation of the hexoses in the 1 position.



Through a series of reactions discussed in detail in the chapter on galactosemia (Chap. 7) galactose 1 phosphate can furnish glucose 1 phosphate and thus glucosyl units for addition to the ends of the outer chains of the glycogen molecule without the intercession of phosphoglucomutase. Supporting this concept [52] are the incorporation studies with galactose  $2\text{-C}^{14}$  and glucose  $2\text{-C}^{14}$  which show less randomization of the label in the glucosyl moieties of glycogen when the substrate provided is galactose than when it is glucose.

It has long been known that liver can produce glycogen twice as rapidly from fructose as from glucose. In traversing the liver little fructose escapes into the general circulation and there is little hyperglucemia [53]. This curious circumstance is probably attributable to the advantages possessed by the specific fructokinase over hepatic glucokinase.

Hers [54] has shown that fructose incorporation into glycogen involves splitting of hexose into trioses and recondensation of the fragments. In vivo hepatic glucokinase activity appears to be relatively weak and this important and critical phase of hepatic glycogen formation and its regulation remain relatively mysterious. If glucose 6-phosphate is the initial product of hepatic kinase activity and if fructose and hexose derived from gluconeogenesis must pass through glucose 6-phosphate on their way to glycogen it is not clear how this glucose 6-phosphate escapes the action of glucose 6-phosphatase. Partition either physical or functional of glycogen formation and degradation becomes an attractive hypothesis.

### *Phosphoglucomutase*

Presumably during conditions of glycogen synthesis sufficient glucose 6-phosphate accumulates within the cell to force formation of glucose 1-phosphate against the unfavorable 19:1 glucose 6-phosphate:glucose 1-phosphate equilibrium of this enzyme.

### *Phosphorylase*

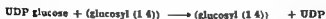
In vitro in the presence of a primer polysaccharide and at pH 7.0 this enzyme will add glucosyl units in  $\alpha$ -1,4 linkage to the outer chains of the primer provided there is more than 1 mole of glucose 1-phosphate per 3 moles of inorganic phosphate in contact with the enzyme. Recent work by Lerner et al. [55] demonstrated formation of glycogen by insulin-stimulated rat diaphragms in short time experiments despite a highly unfavorable ratio of inorganic phosphate to glucose 1-phosphate of 30:1.

### *UDPG-glycogen transglucosylase*

This enzyme discovered in liver by Leloir [9] and detected in muscle by Villar Palasi [56] and others [10, 57, 58] provides a pathway to glycogen independent of phosphorylase. The steps involved may be summarized



The enzyme UDP-glucose:pyrophosphorylase catalyzing this reaction is abundant in liver, red blood cells, mammary gland, and muscle.



This enzyme [11] variously termed UDPG-glycogen transglucosylase and UDP-glycogen synthetase requires a polysaccharide primer. The glucose residue becomes linked  $\alpha$ -1,4 to the polysaccharide. The reaction is strongly activated by hexose 6-phosphate and no intermediary formation of a disaccharide occurs. The liver enzyme can catalyze the transfer of 100  $\mu$ moles of glucose from UDPG per hr per gm tissue and the muscle

enzyme 220  $\mu$ moles. These values comfortably exceed the observed rates of 30  $\mu$ moles and 5 to 17  $\mu$ moles for glycogen formation *in vivo* in the two tissues. The thermodynamics of the reaction are highly favorable for conversion to glycogen and transfers of the order of magnitude of 99 per cent have been observed [10]. Mommaerts et al. [69] envision that normally in muscle glycogen is synthesized at a certain rate by this enzyme but is kept at a moderate level by periodic decreases whenever glycogenolysis increases in association with exercise. When muscle work is performed phosphorylase *b* is activated to phosphorylase *a*. They further postulate that other factors besides the presence of this enzyme are in operation to limit the amount of glycogen which can be deposited in muscle.

The discovery of this enzyme has made possible an understanding of the phosphorylase-deficient type of glycogen deposition disease but it has not entirely clarified the dilemma concerning the incongruities of various circumstances of glycogen breakdown and synthesis alluded to in the discussions of phosphoglucomutase and hexokinases. In effect it has put head back consideration of these problems at least one enzymatic step in that it makes glucose 1-phosphate the key compound in the ebb and flow of glycogen regulation. The further partitioning of the processes of formation and breakdown *in vivo* is still an attractive hypothesis. The activating effect of glucose 6-phosphate on this enzyme is intriguing in that it provides another possible mechanism for regulation of the flow of biochemical events.

#### *Amylo (1 $\rightarrow$ 1,6) transglucosidase*

When a peripheral chain of a growing glycogen molecule reaches between 7 and 21 glucosyl units by means of successive transfers from UDPG (or phosphorylase action) the transglucosidase transfers a string of as yet undetermined number of 1,4-linked glucosyl units from their proximal 1,4 attachment on the main chain to a 1,6 attachment to the same or another unit of the main chain allowing renewed 1,4 additions to the bare end [60]. An arborized structure results and the new 1,6 link constitutes a branch point. The enzyme has been referred to as 'brancher'.

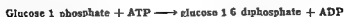
The thermodynamic properties of this enzyme have not been described but it is generally accepted as being irreversible and the specific debrancher amylo-1,6-glucosidase previously discussed is necessary to undo the 1,6 linkage formed (Fig. 6-4).

#### *Accessory Enzymes*

Two enzymes have been found in animal tissues which conceivably could influence the accumulation or dissipation of glycogen by regulating the availability of glucose 1,6-diphosphate for the phosphoglucomutase reaction. The enzymes have not been extensively studied and little

information concerning their physiologic roles if any is available. These are

*Glucose 1 phosphate kinase* [61] which catalyzes the reaction



*Hexose 1 6-diphosphatase* [62] which catalyzes the reaction



## FACTORS REGULATING THE CONCENTRATION OF GLYCOGEN IN THE TISSUES

New details concerning the regulatory factors for tissue glycogen have been added since the comprehensive review by Recant [63]. Since diagnostic procedures and therapeutic attempts have been based on such factors, they will be described briefly.

### *Nutrition*

Liver glycogen is a principal reserve source of blood glucose. Rapid depletion occurs in fasted animals. Diets high in carbohydrate, especially those high in fructose or fructose sources [64], rapidly replete the liver glycogen. Excessive deposition of liver glycogen due to hyperalimentation in the normal subject has not been described. There appears to be a finite limit in the normal animal to the amount of glycogen which can be stored, and when this is reached carbohydrate is diverted to fat synthesis and stored as such in the adipose depots. Approximately 45 gm glucose is available to a fasting man from liver glycogen. Under basal conditions of peripheral glucose utilization, this would not suffice to maintain normoglycemia for more than a few hours unless supplemented by glucose from gluconeogenesis.

In the muscles and other peripheral tissue, any substance which increases glucose uptake from the extracellular fluid and circulating plasma increases tissue glycogen levels, and materials which inhibit glucose uptake have the opposite effect. During sustained vigorous muscle contractions, glycogen is rapidly consumed. This occurs despite enhanced entry of glucose from the blood [65] which also attends muscle work and persists for a considerable time after resting conditions have been re-established, thereby facilitating replenishment of glycogen stores.

### *Insulin*

Experiments employing incubation of the isolated rat diaphragm clearly prove that glucose uptake and glycogen synthesis are strikingly enhanced by insulin [66]. Stadie and coworkers [67] have presented evidence that enhancement of muscle glucose utilization by insulin is primarily achieved by increased glycogen formation.

The problem of a direct action of insulin on hepatic glycogen formation is confusing and in some doubt. Positive effects on net glycogen synthesis *in vitro* have been denied consistently, but enhanced incorporation of radiolabel into glycogen from glucose C-14 supplied in the medium has been observed [68]. Renold et al. [69] have found positive insulin effects *in vivo*. It is well established that inulin therapy in uncontrolled diabetes and in animals experimentally deprived of insulin causes depletion of low liver glycogen stores. The effect is not immediate and takes a matter of hours. This is reminiscent of the time requirement for the adaptive changes in glucose 6-phosphate activity levels. It may be that insulin action on the liver involves adaptive changes in one or more enzymes of glycogen formation and that the effect is not a direct immediate one as it is on the penetration of glucose into muscle cells.

The usual sequence of events following exogenous insulin administration to an intact normal animal is an immediate increase of glucose uptake from the blood by the muscle and increased glycogen in these tissues, the blood sugar falls and liver glycogen is mobilized in defense. Later if an adequate glucose supply is available liver glycogen also rises.

#### *Adrenal Steroids*

The 11 and 11-17-oxysteroid hormones of the adrenal cortex have a profound influence on liver glycogen. Before specific chemical methods were perfected bioassay of these hormones depended on the ability of the test substance to promote storage of liver glycogen in adrenalectomized animals [70]. Since no immediate direct enzymatic effect on hepatic glycogen economy has been described the enhancement of glycogen deposition seems best related to ability of these steroids to increase gluconeogenesis from protein. Recent evidence suggests that this action is probably related to stimulation of hepatic transaminase activity [71]. Other evidence has been presented that these hormones decrease the glucose uptake of muscle and secondarily decrease muscle glycogen synthesis [72].

The combined effects of increasing liver glucose output through stimulation of gluconeogenesis and decreasing peripheral uptake of glucose are thought to account for the ability of these steroids to defend the adrenalectomized animal from hypoglycemia and to produce diabetes mellitus when given or elaborated in excessive amounts. The relatively long lapse of time required for effects to become apparent *in vivo* and the inability to obtain distinct carbohydrate changes *in vitro* suggest that adaptive rather than direct mechanisms are involved.

#### *Somatotropin and Other Pituitary Factors*

In the presence of adequate insulin somatotropin promotes accumulation of liver glycogen, spares carbohydrate from combustion, and favors

protein synthesis and accretion by shifting metabolism toward the mobilization and utilization of lipid. As with glucogenic adrenal steroid, liver glycogen storage is permitted and enhanced and liver glucose output increased but the critical difference in the two influences is that the pituitary factor accomplishes these effects at the expense of fat while the adrenal hormones do so at the expense of protein. An inhibitory effect of pituitary factors on extracted muscle hexokinase has been reported. The prevention of this inhibition by added inulin constitutes the evidence for a useful hypothesis concerning a mechanism of insulin action [73-74].

Glycogenolysis in the beef adrenal cortex is enhanced in vitro by the presence of ACTH in the medium [75]. Haynes has demonstrated that the effect is mediated through an accumulation of adenosine-3, 5 monophosphate within the tissue and an increase in active phosphorylase. The situation appears to be analogous to the activation of liver phosphorylase by glucagon and epinephrine. Haynes suggested that the activation of adrenal cortex phosphorylase by ACTH could be the mechanism by which the pituitary hormone stimulates steroid production for he points out that glycogenolysis would stimulate the glucose-6-phosphate dehydrogenase system and provide an abundance of reduced TPN required for corticosteroid biosynthesis.

*Glucagon and Epinephrine*

Both glucagon and epinephrine are powerful activators of hepatic glycogenolysis and produce significant hyperglycemia when injected into animals. Their glycogenolytic action in the liver is the basis of an important diagnostic test in glycogen deposition diseases. It is a curious paradox that the exact enzymatic mechanisms of action in glycogen metabolism have been better worked out for glucagon than for any other known humoral agent and yet there is no firm hypothesis concerning its physiologic role or importance. The enzymatic changes due to epinephrine as regards glycogen breakdown are identical with those of glucagon with the exception that epinephrine works in muscle enzyme systems as well as in the liver. Glucagon stimulation of glycogenolysis is confined solely to the liver [28] and glucagon possesses none of the vasomotor regulating properties of the catecholamines of the adrenal medulla and sympathetic ganglia. Despite the similarity of the enzymatic mechanisms of action of the two, Cornblith [76] could not obtain additive results when liver slices were incubated in concentrations of the agents which independently give a response of 50 per cent or less of maximum.

Circumstantial but almost conclusive evidence has pinpointed the alpha-cells of the islets of Langerhans as the source of glucagon [77]. If more than 75 per cent of these cells are destroyed by the administration of cobalt salts before a pancreas is extracted the hyperglycemia



producing properties of the extract are lost [78]. Attempts at crystallization of this hyperglycemic factor have been successful [70]. It is a zinc free polypeptide with 29 amino acid components [79a] differing from insulin by containing methionine and tryptophan and by lacking proline isoleucine and cystine found in the latter. The problem of glucagon inactivation was attacked by Goldner et al. [80] by perfusing the material through isolated liver. They reported that passage through the liver is attended by a loss of hyperglycemia provoking activity even though adsorption during passage does not take place. Williams and coworkers [81] have evidence that glucagon, insulin and corticotropin share the same deactivating enzyme system in liver. Hill and Smith [82] have suggested that leucine aminopeptidase detected in the submicroscopic particles of rat liver homogenates is an important enzyme in the degradation of the polypeptide since they were able to demonstrate complete rapid and facile hydrolysis of crystalline glucagon by this enzyme *in vitro*.

The potency of the crystalline material is impressive. 0.7  $\mu\text{g}$  per kg is sufficient to produce a 50 per cent increase in blood sugar. Administration of insulin sufficient to give a fall in blood sugar of the same magnitude requires a twenty five-fold larger dose on a molar basis. Hyperglucemia seldom persists for as long as 60 min following a dose of glucagon. Administration is not attended by tachycardia, blood pressure rise or increased release of lactic acid from the muscles. All these phenomena occur regularly when doses of epinephrine sufficient to provoke equivalent hyperglycemia are used. Further purified glucagon can be given subcutaneously, intramuscularly or intravenously with equal safety and approximately equal effectiveness in contrast to epinephrine nor epinephrine or other sympathomimetic amines. An attendant phenomenon which has been observed is that simultaneously administered sympatholytic agents counteract the hyperglycemogenic actions of epinephrine but not of glucagon [83]. Obviously a positive hyperglycemic response to glucagon or epinephrine presupposes an adequate supply of mobilizable liver glycogen.

The interchange between active phosphorylated liver phosphorylase and inactive dephosphophosphorylase and the depolymerization of stored glycogen has already been described. It is via changes in this interplay that glucagon and epinephrine effect their glycogenolytic action. As a result of the series of brilliant contributions by Sutherland and coworkers [12-14, 23, 24] the mechanisms of glycogenolytic action of these two humoral agents can be described in detail.

1. Incubation of liver with glucagon or epinephrine results in the appearance of a heat stable dialyzable nucleotide containing adenine, ribose and phosphate in molar ratios of 1 : 1 : 1. This has been tentatively identified as adenine ribose 3'  $\rightarrow$  phosphate.

2 In the presence of this cofactor and ATP the proportion of phosphorylase in the active phosphorylated form rises sharply. It is not clear whether this increase in the active form is the result of stimulation of the dephosphophosphorylase kinase activity or of inhibition of the deactivating liver phosphorylase phosphatase.

3 The net increase of active phosphorylase results in an increased rate of glycogenolysis and subsequent increased release of free glucose. Increase in glycogen synthesis as a result of glucagon or epinephrine activation of phosphorylase has not been observed.

4 In the intact animal with normal islet cell function hyperglycemia induces insulin release. The ability of epinephrine to promote muscle glycogenolysis, a phenomenon not shared with glucagon, modifies the insulin release response in the periphery and explains the rise in serum pyruvate levels after epinephrine hyperglycemia but not after glucagon hyperglycemia [84]. This difference in action in muscle also serves as an adequate explanation for the failure to observe a fall in serum inorganic phosphate concentration after epinephrine hyperglycemia. A fall is seen routinely after glucagon hyperglycemia [79].

It is of interest that sympathomimetic amines other than L-epinephrine can reactivate inactive liver phosphorylase and thereby enhance glycogenolysis. Presumably the reactivation with these substances proceeds through the intervention of the same cofactor nucleotide. Table 6-1.

TABLE 6-1 RELATIVE LIVER PHOSPHORYLASE-REACTIVATING ACTIVITIES OF SYMPATHOMIMETIC AMINES *IN VITRO* AND *IN VIVO*

| Sympathomimetic amine | Relative liver phosphorylase assay | Relative animal assay |
|-----------------------|------------------------------------|-----------------------|
| L-Epinephrine         | 100                                | 100                   |
| L-Norepinephrine      | 10                                 | 1                     |
| D-Epinephrine         | 1                                  |                       |
| D-Norepinephrine      | 0.4                                | 0.6                   |
| DL-Amphetamine        | 0.0005                             | 0.0                   |

Let norepinephrine be relative to that of L-epinephrine calculated on a molar basis.

Source: Modified from T. W. Ball et al. [15].

modification from Sutherland's report [13] indicates the relative activities of these amines and their isomers and shows the close correlation of homogenate assays with intact animal assays. This is good evidence that the enzymatic processes studied were the more responsible for the actions of these compounds in normal physiology. The hyperglycemia seen in case of pheochromocytoma is undoubtedly explained by the low but definite glycogenolytic potentialities of L-norepinephrine.

### *Thyroid Hormone*

One of the consequences of excessive L-thyroxine administration is the gradual depletion of liver glycogen and increase in glucose lactate and pyruvate in the blood [85]. Similar changes are seen in patients with hyperthyroidism. The biochemical and enzymatic effects of this hormone are considered elsewhere in this volume (Chap. 9). The most plausible explanation for the influence of L-thyroxine on glycogen metabolism is that the depletion results from draining off of hexose and other carbon sources into the energy production systems of the Krebs cycle which have been rendered inefficient because of the uncoupling of oxidative phosphorylation. The uncoupling of oxidative phosphorylation by L-thyroxine is discussed elsewhere [86] (Chap. 9). The hormone has been tried therapeutically in glycogen deposition diseases, apparently with partial success in some cases.

### *Ovarian Steroids*

Progesterone induces an increase in glycogen content of the stromal cells of estrogen provoked proliferative endometrium as they undergo transition into the secretory and decidual phases of their normal cycle. No information is available as to how this stimulus to glycogen accumulation is biochemically mediated but the phenomenon is cited as an indication that glycogen metabolism in highly specialized tissues may be modified by and in intimate relationship with special regulating influences.

### *Diseases*

The low glycogen content of some cirrhotic livers may be related to declining enzyme concentrations which occur as the disease reaches its advanced states. In diabetes in spite of low liver content when the disease is uncontrolled increased glycogen may be found in the kidney, pancreatic islets and cardiac muscle [87, 87]. Deposition of glycogen in the pigment epithelium of the iris, a tissue which normally has little or no histochemically demonstrable glycogen has also been noted in diabetic patients [88].

Perhaps these anomalous depositions are the result of the hyperglycemia. They serve as suggestions that the glycogen metabolism of specialized tissues must be characterized independently.

## DISEASE STATES RESULTING FROM PRIMARY ABNORMALITIES IN GLYCOGEN METABOLISM

Gerty Cori deplored the practice of treating all glycogen deposition diseases as a single uniform disease; she recommended a classification based upon specific causative enzyme defects [3]. She also pointed out

that naming the entities by the predominant organ of excessive glycogen content leads to misconceptions and expressed a preference for a nomenclature consisting of serially numbered types. While a numbering system allows no criticism on semantic grounds for present purposes it is convenient to modify and extend Cori's classification in order to indicate the specific enzymatic step which is at fault in each of the various glycogen storage diseases which has been elucidated (Table 6-2).

TABLE 6-2 TYPES OF GLYCOGEN DEPOSITION DISEASES (GLYCOGEN TYPE NO. 1)

| Cor<br>typ | Enzyme defect          | Organ    | Glycogen content | Enzyme | Storage site                       |
|------------|------------------------|----------|------------------|--------|------------------------------------|
| 1          | Glycogen 6-phosphatase | Liver    | Normal           | Normal | Classical glycogen storage disease |
| 2          |                        | Glycogen | Normal           | Normal | Glycogen storage disease           |
| 3          | Amylo-1,6-glucosidase  | Liver    | Abnormal         | Normal | Glycogen storage disease           |
| 4          | Amylo-1,4-glucosidase  | Liver    | Abnormal         | Normal | Glycogen storage disease           |
| 5          | Glucose 1-phosphatase  | Liver    | Normal           | Normal | Glycogen storage disease           |
| 6          |                        | Liver    | Normal           | Normal | Glycogen storage disease           |

It should be emphasized that this system of classification is not permanent or necessarily the most advantageous clinically. The classifications of Chaptal et al [89] of Zellweger [90] and of Andersen [91] take more cognizance of variations in clinical manifestations in cases where enzyme defects have not been apparent. Citzelmann [92] in his outstanding and exhaustive review of the glycogen deposition disease problem and its relationship to glucagon prefers a functional classification into two groups: the first with a good response and the second without a normal response to glucagon. At the time his proposal was made all forms of the disease with generalized glycogen storage, normal enzyme, and normal glycogen fell into the glucagon responsive category, and all forms with enzyme deficiency fell into the abnormally responsive category. More recently the description of myophosphorylase deficiency (glycogenosis 1) in which the glucagon response is normal and the enzyme defect and excessive glycogen deposition are present in the skeletal muscles only tends to make the classification by glucagon response confusing and restrictive. Therefore it seems better to follow Cori's system extending it as specific etiologic mechanisms are delineated and

emphasizing differences in specific etiology rather than functional or clinical variations

#### GLUCOSE 6 PHOSPHATASE DEFICIENCY HEPATORENAL GLYCOGENOSIS

Synonyms von Gierke's disease = type 1 of Cori hepatorenal form, hepatonephromegalia glycogenica van Creveld von Gierke's disease

In retrospect it is probable that the examples of children with stunted growth and hepatomegaly described by several authors [93-96] were

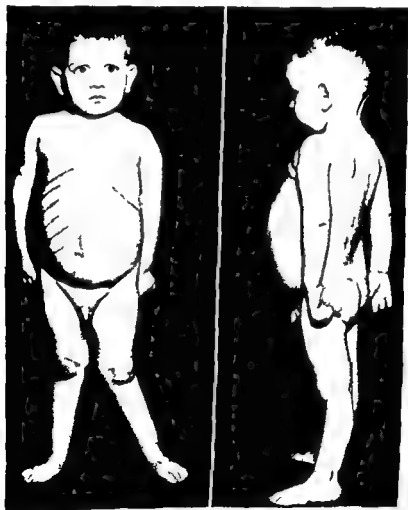


Fig 6-3 G I a 6-year-old patient with biochemically proved absolute deficiency of hepatic glucose 6-phosphatase. The liver contour has been outlined with a marking pencil. Xanthomas present over the elbow knes and buttocks cannot be discerned in the photograph. Note the characteristic lumbar lordosis abdominal contour and sufficiency of depot fat accumulation.

instances of the disease first described pathologically by von Gierke in 1929 [97]. Since von Gierke's description numerous articles and reviews have appeared which have given this disease a full and extensive characterization. The interested reader is referred to Gitzelmann's paper [97] for a complete listing of these authors and the contributions of Bischoff [98], Schonheimer [99], Kimmelstein [100], Wagner [101] and Van Creveld [102-103] especially are recommended.

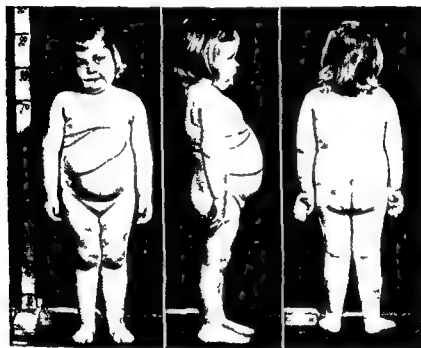


Fig 6-6 P D a 4 year-old patient with chemically proved absolute deficiency of hepatic glucose 6-phosphatase. The accumulation of depot fat appears more than adequate.

Symptoms and recognizable clinical signs usually appear in the first year of life and hepatomegaly may be present at birth. Convulsions due to hypoglycemia, failure to thrive, episodes of precipitous, severe acidosis, and eruptive xanthomas are the most frequent manifestations. History of an older afflicted sibling should arouse suspicion and the disease should be searched for at birth in all subsequent offspring of the same mating.

Physical findings in a typical case usually include (Figs 6-5, 6-6)

1. Retarded growth without disproportion of the head, limbs, or trunk length.

2 Tendency to adiposity with notable accumulations in the depots of the cheeks, breasts, buttocks, and the backs of the arms and thighs

3 Immense abdominal rotundity especially noticeable in nurrlings and small children due to a massively enlarged liver. The blunt hard edge may extend as low as the level of the iliac crest. The spleen is not palpable and the liver not tender in typical cases

4 The skin usually has a yellowish and pilled hue and pinkish yellow xanthomas on the elbows, knees or buttocks may be present [10]

5 The gait is typically swinging and on a wide base with at times accentuation of lumbar lordosis in order to accommodate the massive liver and abdomen

6 If abdominal examination can be adequately performed enlargement of the kidneys may be apparent

Laboratory examinations in cases of some duration reveal many or all of the following findings

1 Marked and at times profound fasting hypoglycemia (0 to 15 mg per 100 ml true blood glucose). In many cases there appears to be a clinical tolerance to hypoglycemia, perhaps because of some "adaptation." One is frequently amazed by the absence or paucity of clinical signs and symptoms of hypoglycemia in the presence of blood glucose concentration reduced to a point where convulsions and unconsciousness would be expected in a normal child. Normal electroencephalograms were found in two cases studied by Schulman and Saturen [10] in the presence of very low blood sugar levels. Postcibal blood sugar levels tend to be high and values in the first hour after a carbohydrate feeding may be in the diabetic range

2 Marked hyperglyceridemia, hypercholesterolemia and hyperfatty acidemia. Frequently the content of fatty solids in the serum reaches 10 per cent or more by volume. Indeed a proportional correction must be made in order to determine the true aqueous concentrations of various dissolved components of the serum. For example in a case where the total plasma solids were approximately 15 per cent by volume application of a correction factor of 1.18 was necessary; this factor when applied to the determined serum sodium concentration of 119 mEq per liter yielded a perfectly normal value of 140 mEq per liter. If this volume correction is not taken into account when hyperlipemia is massive false impressions of depressed electrolytes, NPN,  $\text{CO}_2$ , etc. will be obtained from direct serum determinations (Fig. 6.7).

3 Acetonuria and acetonemia. These are inconsistently present and appear to be related to episodes of acidosis and fasting. It should be mentioned that several cases have been seen in which severe life threatening acidosis supervened without the appearance of significant acetonemia or acetonuria; the absence of ketone bodies therefore cannot be used as reliable assurance that acidosis is not present or impending.

4 Mild anemia in the range of 80 gm per 100 ml has been seen in several cases. Extensive hematologic investigation by McGovern [106] in one case failed to define a causative mechanism.

5 Elevations of serum lactate and pyruvate levels have been frequent in some cases even when acidosis was not present and although they rose significantly in one case when acidosis occurred the additional amount was much too small to account for the bicarbonate which had been displaced.

6 Absolute depression of the serum inorganic phosphate and normal serum alkaline phosphatase activity are frequently observed and may correlate with the generalized decreased bone density encountered on roentgenographic examination of the skeleton. The effect of chronic metabolic acidosis may also play a role in this finding.

Mushrooming of the metaphyses and small epiphyseal growth centers also has been described. A feature of one patient at age 6 was the repeated occurrence of leg fractures following minimal trauma and markedly delayed healing.

7 Glucosuria and a nonspecific aminoaciduria may occur and at times may be massive [107]. It has been suggested that the severity of this finding correlates with the severity of glycogen infiltration of the renal tubules. Aside from this abnormality, it is surprising that even with much enlargement of the kidneys little impairment of renal function can be detected.

8 Liver function tests other than those of carbohydrate metabolism fail to show much deviation from the normal. The albumin globulin ratio is depressed in only about half the cases. Electrophoretic studies [82] in three cases showed increases in  $\alpha_1$  and  $\beta$  globulins along with increases in  $\alpha_2$  and  $\beta$  lipoprotein and  $\alpha$ -mucopolysaccharide.

9 The electrocardiogram is usually normal but may show changes during episode of profound hypoglycemia [108].

10 Increases in the fraction of nonfermentable reducing substances in the whole blood have been reported [109] and were shown to be due to accumulation of phosphorylated glycolytic intermediate chiefly glucose 6-phosphate, fructose 6-phosphate



Fig 6—Hematocrit determination on patient at 11 following intense hypoglycemia of serum dilution. Hematocrit. The total plasma solids were approximately 1 per cent by volume at the time of the determination.



and hexose diphosphate. Elevations of total glycogen content of whole blood were also noted. Apparently these substances are in the circulating blood cells since it has not been possible to detect their presence by specific enzymatic assay in the serum [57].

### *Functional Tests*

Further useful information in a suspected case of this disease can be obtained by performing functional tests of carbohydrate metabolism. The glucose tolerance test may show an abnormally high rise and in some cases a delayed fall. Caution should be used in performing this test in that an overnight fast may provoke hypoglycemic convulsions or unconsciousness. Fructose and galactose tolerance tests are said to give flat or normal curves. In a relatively compensated child with this disorder nitrogen balance is usually normal. Insulin tolerance tests are to be avoided in view of inability of the patient to defend himself against hypoglycemia.

Most important are the responses to the glycogenolytic properties of glucagon and epinephrine. By the mechanisms previously described in the section on liver phosphorylase activation, administration of these agents in a well nourished normal child with a well glycogenated liver results in 40 to 60 per cent rise in blood glucose within 10 to 20 min by virtue of the depolymerization of stored glycogen to glucose 1-phosphate subsequent conversion to glucose 6-phosphate and ultimate release into the hepatic vein blood after dephosphorylation of free glucose and inorganic phosphate. In biochemically verified examples of this disease the hyperglycemic response to these agents is abolished or decidedly diminished. The severe hypofunction of glucose 6-phosphatase found in this disease limits the amount of glucose 6-phosphate which can be deesterified even in the face of a brisk rise in intracellular glucose 6-phosphate concentration.

It will be recalled that as much as 8 per cent of the glucose of a glycogen molecule can be released as free glucose by debrancher action (amylase 1-6-glucosidase) without need of the specific phosphatase. Accordingly some increase in peripheral blood glucose is to be expected even in the total absence of glucose 6-phosphatase. This is a reasonable explanation for the clinical observations that in a few cases of what appear to be classical glucose 6-phosphatase deficiency disease only slightly diminished or approximately normal responses have been found [110]. Partial responses might also be expected in those cases in which glucose 6-phosphatase activity is less severely diminished.

It is conceivable that near normal responses to the two hyperglycemic factors which have been observed in cases thought to have been glucose 6-phosphatase deficiency disease on clinical grounds resulted from con-

fusion with debrancher deficiency limit dextrinosis (see Amylo-1 G glucosidase above). In the latter condition after a recent feeding there may be considerable transient elongation of the terminal branches of the glycogen molecule which makes possible a substantial rise in blood sugar when depolymerization is promoted by increasing active phosphorylase. In some cases direct biochemical investigation of liver tissue in regard to glycogen structure and the activity levels of glucose 6-phosphatase and amylo-1 G glucosidase is necessary in order to differentiate the two conditions with absolute certainty.

Suffice it to say that marked response to the hyperglycemic actions of glucagon and epinephrine is a finding lending strong support to the diagnosis of hepatic glycogenosis but a normal response does not absolutely rule it out. Of the two the glucagon test is the more useful because of avoidance of the vasomotor effects of epinephrine and the occasional confusing interference with muscle carbohydrate metabolism. Since nonantigenic and nonpyrogenic preparations of glucagon are now available in a lyophilized form for long storage and easy solubilization it is customary to give 10 mg crystalline glucagon intravenously to a subject after an 8-hr fast. Determinations of fasting 10- 20- 30- 40- and 60-min true blood glucose are made. When it seems advisable the duration of the preliminary fast period is reduced.

Another functional test useful in diagnosis was suggested by Schwartz et al. [111]. One gram of galactose per kg body weight is infused intravenously over a 3 min period and preinjection 1- 10- 20- 30- 40- and 60-min blood samples are obtained and analyzed for glucose and reducing sugars.

Hers and Malbrun [112] have shown that fructose 0.5 gm per kg body weight can be used in stead of galactose. In a normal child an elevation of blood glucose occurs after such infusion but in glucose 6-phosphatase deficiency disease the inability of the liver to decarboxylate glucose 6-phosphate formed by intrahepatic conversion of the administered sugar results in a failure of blood glucose to rise. The occurrence of hyperglycemia after such a test implies quantities of glucose 6-phosphate in the liver sufficient for the needs of the organism. Normal hyperglycemic responses with this test have been obtained in debrancher deficiency limit dextrinosis [113] which is the expected response in that no defect in the critical phosphorylase is present. The two conditions can be distinguished on this basis when other test and manifestations are equivocal. The stimulation of galactose and fructose and their interconversion to glucose 6-phosphate by the liver is normal in the uncomplicated case of glycogenosis. However the utilization of galactose is more easily disturbed than is the utilization of fructose when either form of liver disease are present. Caution with the infusion of fructose is also necessary.

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because precipitous production of severe acidosis has occurred with this procedure in an enzymatically verified case of absolute glucose 6-phosphatase deficiency disease [47]

### *Biochemical Studies*

The ultimate and only conclusive diagnostic procedure in glycogen deposition diseases is liver biopsy and direct biochemical study of the amount and structure of the glycogen along with assay of the levels of activity of the specific enzymes. Sufficient tissue for such studies may be obtained by needle aspiration of the liver but since the procedure is a blind one most authorities prefer general anesthesia and surgical biopsy under direct vision. The surgical approach obtains sufficient tissue for pathologic examination and diagnostic information is provided even if glycogen deposition disease is not present. The principal hazard in surgical biopsy is the possibility of fatal outcome due to effects of general anesthesia on a glycogen infiltrated myocardium. Occasionally such infiltration gives no clinical evidence of its existence as part of a generalized glycogenosis or limit dextrinosis. The interested reader is referred to the paper of Hers [5] for a detailed description of the elegant methodology which he has worked out for the required enzymatic studies.

The biochemical findings from enzymatic studies on liver and kidney tissue from examples of glucose 6-phosphatase deficiency glycogenosis were first reported by Cori and Cori [1, 118] and have been amply confirmed by others [8, 119]. The following findings can be considered diagnostic:

1. Glycogen content above 4 per cent by wet weight.
2. Normal structured glycogen as determined by alternate phosphorylase and amylo-1,6 glucosidase stepwise degradation viz. normal chain length and normal frequency of branch points.
3. Normal or only slightly reduced levels of activity of phosphorylase, amylo-1,6 glucosidase, phosphoglucomutase, UDPG glycogen transglucosylase, and amylo-(1,4  $\rightarrow$  1,6) transglucosidase. The high content of glycogen makes it desirable to compare enzyme activities not only per unit weight but also per unit of deoxyribonucleic acid or per cell.
4. Absent or very low glucose 6-phosphatase activity. Hers expresses the opinion [8] that only total absence of the enzyme can be held responsible for the disease. In other forms of hepatic disease moderately reduced glucose 6-phosphatase activity may be due to absolutely reduced content of the enzyme or dilution of protein content by fat or other infiltrations. Apparently total or almost total absence of this enzyme is necessary for glycogen accumulation when other glycogenolytic enzymes are present at adequate activity levels. In the experience of Hers [8] accumulation of glycogen can be attributed to other enzymatic deficiencies when glucose 6-phosphatase assays show only moderate scarcity of the enzyme.

Hers further pointed out that total or severe reduction of glucose 6-phosphatase is required to impair the hyperglucemia following galactose or fructose infusion.

5 The total fat content of the liver is frequently much increased but this finding is not invariable and is not a *sine qua non* for the biochemical diagnosis. Increased renal fat content does not seem to occur but glycogen deposition with increase in organ size may be extensive and total absence of glucose 6-phosphatase can be demonstrated.

Microscopic findings are not diagnostic and consist of an abundance of glycogen granules in hepatic cells and in the cells of the convoluted tubules. Fatty vacuolization of many liver cells and the variability of glycogen content from cell to cell combined with central placement of the nuclei give these cells a plant like swollen appearance when viewed in hematoxylin and eosin preparations. In the typical case fibrosis, ductile proliferation and destruction of lobular architecture characteristic of a cirrhotic process are not found.

The epithelial cells of the convoluted tubules often protrude into the lumen to produce a scalloped effect. The cells of the loops of Henle show similar but less pronounced changes while the collecting tubules are usually normal. Cellular debris found in the lumens contains some glycogen and may be in the form of cysts. In some cases increase in interstitial fibrous tissue has been described.

The histochemical technique of Chiquoine [116] beautifully demonstrates the distribution of glucose 6-phosphatase within the cytoplasm of normal hepatic cells and documents the absence of this enzyme in normal nuclei and stromal elements. It also shows that there is a decreasing gradient of enzymatic activity extending from the periphery of the normal liver lobule toward the central vein. In cases of glucose 6-phosphatase deficiency, glycogenosis, this same technique can be employed to demonstrate in a most dramatic and graphic manner the absence of the enzyme (Fig 6-8).

Best's stain can be employed to confirm large deposits of glycogen in the liver and kidneys and only small amounts in the heart, tongue and skeletal muscle [116].

The hyperlipidemia in which all fractions of the blood lipids participate is a characteristic finding in biochemically verified cases of glucose 6-phosphatase deficiency, glycogenosis. Because the high level of serum lipids and lipoproteins in this condition grossly resemble those seen in uncontrolled diabetes mellitus, it has been suggested that the stimulus to hyperlipidemia may be the same in the two diseases. By the same analogy, massive stimulation of ketogenesis has been an attractive hypothesis in accounting for the proclivity to severe acidosis in glucose 6-phosphatase glycogenosis. However, there are some impressive incongruities in the two conditions which indicate that much more study

because precipitous production of severe acidosis has occurred with this procedure in an enzymatically verified case of absolute glucose 6-phosphatase deficiency disease [47]

### *Biochemical Studies*

The ultimate and only conclusive diagnostic procedure in glycogen deposition diseases is liver biopsy and direct biochemical study of the amount and structure of the glycogen along with assay of the levels of activity of the specific enzymes. Sufficient tissue for such studies may be obtained by needle aspiration of the liver but since the procedure is a blind one most authorities prefer general anesthesia and surgical biopsy under direct vision. The surgical approach obtains sufficient tissue for pathologic examination and diagnostic information is provided even if glycogen deposition disease is not present. The principal hazard in surgical biopsy is the possibility of fatal outcome due to effects of general anesthesia on a glycogen infiltrated myocardium. Occasionally such infiltration gives no clinical evidence of its existence as part of a generalized glycogenosis or limit dextrinosis. The interested reader is referred to the paper of Hers [8] for a detailed description of the elegant methodology which he has worked out for the required enzymatic studies.

The biochemical findings from enzymatic studies on liver and kidney tissue from examples of glucose 6-phosphatase deficiency glycogenosis were first reported by Cori and Cori [1, 113] and have been amply confirmed by others [8, 114]. The following findings can be considered diagnostic:

- 1 Glycogen content above 4 per cent by wet weight
- 2 Normal structured glycogen as determined by alternate phosphorylation and amylo-1-6-glucosidase stepwise degradation viz normal chain length and normal frequency of branch points
- 3 Normal or only slightly reduced levels of activity of phosphorylase, amylo-1-6-glucosidase, phosphoglucomutase, UDPG glycogen transglucosylase and amylo-(1-4  $\rightarrow$  1-6) transglucosidase. The high content of glycogen makes it desirable to compare enzyme activities not only per unit weight but also per unit of deoxyribonucleic acid or per cell.
- 4 Absent or very low glucose 6-phosphatase activity. Hers expresses the opinion [8] that only total absence of the enzyme can be held responsible for the disease. In other forms of hepatic disease moderately reduced glucose 6-phosphatase activity may be due to absolutely reduced content of the enzyme or dilution of protein content by fat or other infiltrations. Apparently total or almost total absence of this enzyme is necessary for glycogen accumulation when other glycogenolytic enzymes are present at adequate activity levels. In the experience of Hers [8] accumulation of glycogen can be attributed to other enzymatic deficiencies when glucose 6-phosphatase assays show only moderate scarcity of the enzyme.

glucose 6-phosphate which would normally have been released after deesterification as free blood glucose but instead is channeled through the Embden Meyerhof pathway. When the capacity of the Krebs cycle to accept and further dismutate the three carbon fragments has been exceeded, pyruvate and lactate are delivered into the hepatic outflow. Theoretically, the hexose monophosphate oxidative pathway for disposition of glucose 6-phosphate would also be operating at maximal activity under the stimulus of a much increased substrate load. These circumstances if present would be highly favorable for increased hepatic synthesis of fatty acid. Therefore it is conceivable that hyperlipidemia in this condition is the result in part of overproduction in the liver rather than overmobilization from the depots.

The theoretical considerations of the hyperlipidemia and the genesis of acidosis as they occur in this disease make it clear that faulty biogenetics involving the lack of one critical enzyme has provided an individual in whom by means of suitable investigation elucidation of the in vivo interdependence and mechanisms of regulation of hepatic carbohydrate and lipid metabolism may be possible.

### Genetics

Extensive information concerning the genetics of this form of glycogen deposition disease is not available. It has long been recognized that there is a marked frequency of occurrence in siblings and in offspring of consanguineous matings and that the defect is detectable at or shortly after birth in an equal male-female distribution. When the glucose 6-phosphatase deficiency was discovered there followed general acceptance that the mode of transmission is as a completely recessive trait involving a single autosomal gene [117] which endows the liver and kidney with the capacity to synthesize glucose 6-phosphatase. As such this form of glycogenosis qualifies as an example of an inborn error of metabolism.

Until recently detection of the carrier state was not possible since severe deficiencies of the enzyme are necessary before glycogen deposition, hypoglycemia and secondary metabolic consequences occur. Hsia and Kot [118] have studied the phosphorylated intermediates of the glycolytic cycle of the red cells of affected persons and their parents. Their results show a statistically significant increased content of these compounds in the presumed heterozygotes when compared with normal controls. Although the cause of increased phosphorylated glycolytic intermediate content in the affected homozygotes themselves is not understood, the data indicate that the degree of increase is of approximately the same magnitude in the heterozygotes. These findings tend to confirm that this disease is a simple recessive trait (Table 6-3).

Information concerning the appearance of glycogen and enzymes necessary for its metabolism during the development of the guinea pig



of the genesis of hyperlipidemia and acidosis is necessary before a hypermobilization theory can be accepted. For example, one cannot help being impressed with the relative overabundance of stored adipose tissue in a child with this defect. Since this occurs when hyperlipidemia is chronically massive, excessive mobilization of the mechanism would not eventual depletion of adipose depots rather than relative excess be

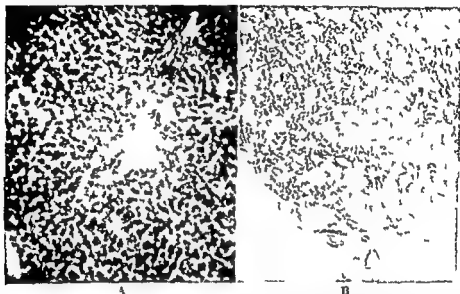


Fig. 6-8 A Distribution of glucose 6-phosphatase in 10- $\mu$  frozen section of liver of a case of idiopathic generalized glycogenosis (type I). The enzyme is present only within the cytoplasm of the hepatic cells and is absent from all nuclei and stromal elements. This appearance corresponds with that seen in normal liver. Note the gradient of enzymatic activity extending from the periphery of the lobule to the central vein (light microcopy 85 $\times$ ). B Section of the liver from a case of glucose 6-phosphatase deficiency glycogenosis (type I). No enzymatic activity can be demonstrated (phase contrast microscopy 100 $\times$ ).

more likely? The supply of glucose to the periphery is sharply curtailed by the hepatic enzyme deficiency. What then is the primary substrate transported from the alimentary influx via the liver to the periphery for depot lipid synthesis?

Although excessive glycogen deposition in the liver can account for disposal of some alimentary carbohydrate through sequestration, there appears to be a finite limit to how much the hepatic glycogen content can be increased. Some other pathway of disposition of carbohydrate carbon in the liver seems probable. Some of this carbon apparently is discharged from the glucose 6-phosphatase-deficient liver by increasing the production and release of pyruvate and lactate, since elevated blood levels are routinely found. Presumably this is the fate of some of the

Another puzzling question involved in fetal glycogen synthesis is that of the source of the initial primer molecules of the polysaccharide for both UDPG glycogen transglucosylase and phosphorylase as found in the animal after birth require the presence of such a primer for their synthetic action. It is not known whether primer is transferred with the gametes or if there is a special system for its synthesis in fetal life. It is also possible that these two enzymes can function without primer requirement during this period or that the initial primer is of maternal origin.

### *Therapy*

The question of therapy in glucose 6-phosphatase deficiency disease should not be regarded as hopeless. With recognition of the metabolic mechanisms involved in the clinical manifestations it has been possible through relatively simple rational therapy to prevent death in early life. It has been suspected that the disease becomes less severe in those who survive beyond the fourth year although hepatomegaly and severe reduction of enzyme activity remain unchanged [121].

During infancy severe hypoglycemia with its inherent threat of damage to the central nervous system is the central clinical problem. A reasonable and effective approach to this problem is the institution of frequent feedings of carbohydrate throughout the day and night. On theoretical grounds it seems advisable to avoid a great excess of carbohydrate. The aim is to provide a relatively constant influx of blood glucose from alimentation without encouraging rapid increase in glycogen deposition. The creation of a portocaval shunt might be very effective in this regard but no report of a trial of this procedure has been found.

Since infection and acidosis are the usual proximate causes of death it behooves the clinician (1) to protect the child from them in so far as is possible and (2) to recognize their appearance quickly and institute corrective therapy. Rehydration intravenous glucose vigorous antibiotic therapy if infection is found all employed with care and frequent laboratory determinations give results comparable to those in the treatment of diabetic ketoacidosis provided the situation has not deteriorated too far. Administration of bicarbonate in suitable amount is effective in hastening the reversal of acidosis. Alkali in the form of lactate seems inadvisable in view of the hyperlactic acidemia and the possibility of further exceeding the capacity for its utilization.

The above methods of therapy although effective in preventing dangerous hypoglycemia and loss of life due to acute episodes of acidosis are strictly defensive and do not attempt to correct influence or by pass the underlying enzymatic defect. Attempts to do so with pancreatic extracts hypophyseal extracts adrenocortical hormones epinephrine whole liver liver extracts choline cholic acid lecithin bile salts and

embryo indicate that the mechanisms of genetic expression concerned in this area of metabolism are not simple and that secondary modifying influences may be at work. According to Nemeth and coworkers [119]

TABLE 6-3 RED BLOOD CELL CONTENT OF PHOSPHORYLATED GLUCOSE AND FRUCTOSE IN NORMAL SUBJECTS, PATIENTS WITH GLUCOSE 6-PHOSPHATASE DEFICIENT CYCLOPENOSES, AND THEIR PARENTS

|              | Normal subjects |           | Affected patients |           | Parents<br>(heterozygotes) |           |
|--------------|-----------------|-----------|-------------------|-----------|----------------------------|-----------|
|              | G 6-P           | F 6-P     | G 6-P             | F 6-P     | G 6-P                      | F 6-P     |
| Number       | 10              | 10        | 4                 | 1         | 8                          | 8         |
| Mean         | 0.18            | 0.35      | 1.07              | 1.10      | 1.10                       | 1.06      |
| Range        | 0.05-0.34       | 0.12-0.40 | 0.80-1.00         | 1.15-1.14 | 0.70-1.00                  | 0.86-1.00 |
| (vs. normal) |                 |           |                   |           | 0.33                       | 0.01      |

Expressed as gm per 100 gm red blood cells

SOURCE: Modified from D. V. Hors et al. [118]

there are three distinct phases during the 66-day gestation period of the guinea pig embryo

1 Complete absence of liver glycogen until the fifty seventh day of gestation. All enzymes concerned with synthesis can be demonstrated during this phase except amylo-(1,4  $\rightarrow$  1,6) transglucosidase, the brancher enzyme.

2 Rapid accumulation of glycogen from the fifty seventh day until term, at which time the content of the fetal liver is two or three times that of the maternal liver. Onset of this phase coincides with the appearance of brancher activity, and it has been suggested that its presence initiates the accumulation.

3 Glucose 6-phosphatase activity cannot be detected in the fetal liver until birth [120] when there is a rapid depletion of the previously stored glycogen.

Thus, until birth, the glycogenolytic enzymatic constitution of the normal fetal guinea pig liver appears to be the same as that seen in the disease under discussion. It appears, then, that the expression of the genetic abnormality in glucose 6-phosphatase deficiency disease involves a failure to provide active enzyme at birth and that the absence of the enzyme during fetal development is of no consequence. Information regarding the mechanisms which determine its appearance in the normal animal at birth might have great bearing on the question of a definitive therapy for the deficiency disease. The possibility that the failure of enzyme appearance is secondary to some as yet undetected hormonal abnormality cannot be denied but seems unlikely from current knowledge of biochemical genetics in bacteria and less complex organisms.

colytic pathway at an increased rate. By giving glucose infusions Lowe and coworkers deduced that the hypoglycemia *per se* was not the cause of the hyperlactic acidemia since under this circumstance the lactic acid level decreased. They also found that glycogen mobilization to lactic acid from liver tissue obtained from this patient could be stimulated *in vitro* by glucagon but not by epinephrine and they ruled the question as to whether the currently held theory of identity of glucagon and epinephrine action in regard to glycogenolysis is correct. There was rapid incorporation of glucose  $C^{14}$  into the liver glycogen indicating that synthesis is via the UDP-Glc glycogen transglucosylase continued despite simultaneous mobilization via phosphorylase.

It would appear then that greater amounts of ingested glucose can escape sequestration in liver glycogen if glycogenolysis can be stimulated even though the products of such stimulation must be disposed of as lactate and that substantial reductions in the amount of stored glycogen can be effected with chronic stimulation and disposition via this pathway.

The approach to therapy with L-thyroxine and chronic glucagon administration deserves intensive investigation not only because clinical improvement seems obtainable but also in order to clarify knowledge concerning the interendocrine regulations and interrelationships in hepatic glycogen and carbohydrate metabolism.

#### IDIOPATHIC GENERALIZED GLYCOGENOSIS

Synonyms Pompe's disease type 2 of Cori cardiac form of generalized glycogenosis neuromuscular form of generalized glycogenosis cardiomegalia glycogenica diffusa generalized glycogenosis

It is difficult to draw a dividing line in clinical cases between the cardiac and neuromuscular forms of this disease. Because no differential biochemical criterion has been found the entity will be discussed as a single one with the qualification that in certain examples the involvement of the heart predominates while in other cases the nervous system is more severely afflicted.

Extensive reviews of this condition have been presented by di Sant'Agnese [128-129] and by Gitzelmann [92]. Ordinarily symptoms begin between the second and sixth months of life but may be present from birth. Vomiting, anorexia, failure to grow, weakness of the musculature, drooling, and later cyanosis and dyspnea are the most frequently observed symptoms and signs. Occurrence in siblings is not uncommon.

Physical findings may include

1. Appearance of imbecility not unlike that seen in cretinism or mongolian idiocy. At times enlargement of the tongue occurs (Fig 6-9).
2. Hypotonia of the muscles may be extreme and lead to confusion with amyotonia congenita [130].

roentgen irradiation have all proved fruitless [122]. Several authors have claimed benefit from high protein diets of one sort or another [123] while Bulgarelli [124, 125] has been improved with ACTH and growth hormone when combined with such diets. If a patient has biochemically proved absence of glucose 6-phosphatase it is difficult to understand how increasing protein intake or stimulating gluconeogenesis could improve the situation in regard to maintenance of sufficient blood glucose. Glucose 6-phosphate appears to be an obligate intermediate in the conversion of protein to blood sugar by the liver. On the other hand in debrancher deficiency limit dextrinosis where glucose 6-phosphatase activity is adequate there is no difficulty in releasing glucose 6-phosphate. In the latter disease such maneuvers should be highly effective on theoretical grounds for they exploit the time factor necessary for intrahepatic conversion of protein to carbohydrate and allow prolonged hepatic glucose release. These considerations reemphasize the necessity of distinguishing the two defects by direct enzymatic analysis of liver tissue.

Two other therapeutic approaches to influencing intermediary metabolism are very interesting and appear to have been met with some measure of clinical benefit. Kowliker and Pickering [126] administered L-thyroxine to three patients over a 6 week period and noted quicker rises in blood sugar after oral feedings, decline in the unfermentable fraction of the total reducing substances of the blood, improvement in the responses to glucagon and epinephrine and a diminution of insulin sensitivity. Gitzelmann [92] employing both glucagon and L-thyroxine therapeutically confirmed these results in one case and expressed the opinion that cases in which a good hyperglycemic response to glucagon is present before therapy are the ones most likely to respond favorably. Although no hypothesis as to the metabolic mechanisms by which these effects are achieved has been offered, the well known action of thyroxine in uncoupling oxidative phosphorylation in the Krebs cycle could be the explanation for its effect. Even though it is energetically inefficient and wasteful, increasing the rate of revolution of the Krebs cycle through uncoupling would increase the rate of disposition of carbohydrate and allow reduction in glycogen deposition.

Lowy and coworkers [127] reported a dramatic acute decrease in liver size in a patient with this disease given intensive glucagon therapy. Reenlargement of the organ occurred when therapy was stopped. When glucagon was given immediately after feedings, postprandial glycogen deposition was inhibited and there were greater increases in blood sugar than had been obtained with similar feedings without glucagon. Glucagon injections during fasting hypoglycemia did not raise the blood sugar but produced sharp rises in blood lactate. This suggests that the products of an increased glycogenolysis were being channeled through the gly-

Laboratory examinations make differentiation of this disease from glucose 6-phosphate deficiency and the other enzyme-deficient syndromes relatively easy.

1 Fasting blood sugar, glucose tolerance, galactose tolerance, glucagon and epinephrine responses are all normal. Acetonuria is not found.



Fig 6-10 Roentgenogram of the chest taken at age 10 months in case of H. Gitzelmann showing generalized enlargement of the globular heart with a wide overlying thymus shadow. The appearance is typical of idiopathic generalized glycogenosis (Hyperplasia of R. Gitzelmann [9]).

2 The gross hematologic picture is normal, but staining of the circulating leukocytes for glycogen will reveal massive glycogen deposition and can unmask the diagnosis.

3 A globular cardiac silhouette is a common finding on roentgenographic examination of the chest (Fig 6-10).

4 Electrocardiographic changes such as depressed ST segments, inverted T waves, or less commonly a high peaked T wave may be encountered (Fig 6-11).

Diagnosis can be definitively established by muscle biopsy, glycogen structure analysis, and enzymatic assays.

Histologic pathology [129] consists of marked myocardial infiltration with glycogen which may give a distinctive lacework appearance.



**Fig. 6-9** Upper: A K. at age  $5\frac{1}{2}$  months, a case reported by R. Gitzelmann of idiopathic generalized glycogenosis. An older sister died at the age of  $4\frac{1}{2}$  with the autopsy findings of diffuse cardiac glycogenosis with lesser involvement of liver, kidneys and muscles. Note resemblance to cretinism.

Lower: Same patient at age 9 months. Death occurred at age  $10\frac{1}{4}$  months with cardiac and circulatory insufficiency. At autopsy generalized glycogenosis was found but the glycogen structural analysis and enzyme activity determinations were normal as performed by Dr. G. T. Cori. (By permission of R. Gitzelmann [9].)

3. Enlargement of the heart can usually be detected by percussion and palpation and an apical systolic murmur is not uncommon.

4. Hepatosplenomegaly is only very rarely observed.

5. Various neurologic deficits may be present when involvement of the nervous system is severe [131].

No reasonable explanation of the abnormality in glycogen metabolism in this disease is currently available although there has been speculation concerning a disturbance of hormonal regulation [92] No treatment of benefit is known and it is unlikely that any will be forthcoming until a better biochemical understanding of the pathogenesis becomes available

#### DEBRANCHER DEFICIENCY LIMIT DEXTRINOSIS

Synonyms Forbes disease type 3 of Cori, limit dextrinosis

As previously mentioned it is probable that many cases described in the early literature as von Gierke's disease were examples of this defect rather than glucose 6-phosphatase deficiency glycogenosis In her last publication on the subject Cori [8] stated that 9 cases in all had been studied in her laboratory In Hers series [8] of 15 cases of glycogenosis 5 were of this variety as compared with 7 of the glucose 6-phosphatase deficiency variety Hers ventures the speculation that one of the two cases originally reported by van Creveld which developed favorably may have had amylo-1,6-glucosidase deficiency since muscle glycogen was elevated Hers perfection of the assay methods for amylo-1,6-glucosidase and a clearer understanding of the differentiating metabolic pathophysiology should make confusion of the two diseases unlikely in the future

Descriptions of the clinical manifestations of this disease are not abundant but in general they are very similar to those of glucose 6-phosphatase deficiency except that they tend to be milder and difficulties with hypoglycemia and development are less severe Glycogen deposition in the heart and skeletal muscles which does not occur in the glucose 6-phosphatase deficiency disease can give rise to a somewhat different set of clinical manifestations

In the original case described by Forbes [133] abdominal enlargement had been found at age 1 year The child had a desire for sweets and breadstuffs Acetone was noted in the fasting urine at the age of  $3\frac{1}{2}$  years At this time fasting blood sugars ranged between 34 and 50 mg per 100 ml serum cholesterol was 284 mg per 100 ml and there was a diminished hyperglycemic response to epinephrine The liver reached the iliac crest Subsequent development was normal and the abdominal enlargement decreased During the twelfth year the fasting blood sugars gradually reached higher levels and the serum cholesterol fell Hepatic enlargement was more prominent and marked Bromsulphalein retention was found along with an abnormal cephalin flocculation test Previously liver function tests had been normal In 1955 Recant [63] described the patient then 13 years old as appearing well A certain amount of muscle flaccidity was noted

Histologic findings in the liver biopsy at age 12 included increased fat



because of displacement of the cytoplasm by the mass of glycogen and the nuclei lying free in the center of the cell. Extensive deposition of variable degrees has been noted in all muscle—both smooth and striated—with tongue and diaphragm frequently the most severely affected. Deposits have also been observed in the reticuloendothelial system: kidney cells, liver cells, lymphoid cells, cortical and medullary cells of the adrenal glands, acinar, ductal and islet cells of the pancreas and also in the cells of bone marrow, lymph nodes and thymus. Glycogen accumulation does not cause any reaction on the part of the infiltrated tissue [132].

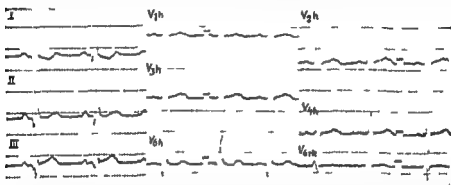


Fig. 6-11. Electrocardiogram taken at age 10 months in case V. K. of Ritzelmann interpreted as showing left ventricular hypertrophy and endocardial damage. Such findings are not uncommon in idiopathic generalized glycogenosis. (By permission of R. Ritzelmann [132].)

In some cases infiltration with a basophilic substance interpreted as mucoprotein or mucopolysaccharide as well as excessive glycogen deposition has been noted in the skeletal muscle [90, 131].

Biochemical examinations, though documenting heart muscle glycogen concentrations as high as 10 per cent by wet weight, have so far failed to detect any abnormality of glycogen structure or aberration of activity of any of the enzymes known to be concerned with its metabolism [9]. Spontaneous glycogenolysis in biopsy specimens proceeds rapidly and suggests that search for an enzymatic cause if one exists should be directed elsewhere than to the glycogenolytic systems.

The central clinical problem in this disease is that of cardiac failure with tachycardia and edema. In a few cases death ensues from aspiration pneumonia and progressive weakness of the muscles of respiration. Death from one or another of these mechanisms within the first year is the rule.

The disease is definitely familial in occurrence. Ritzelmann [92] found 12 afflicted individuals out of a total of 21 children of seven families described in the literature. The mode of inheritance appears to be through a single recessive autosomal gene.

residues which were accumulated between it and the original proximal branch point. The ability to deesterify glucose-1-phosphate and release it to the blood stream as free glucose is not impaired since sufficient glucose 6-phosphatase is present. This explains why gluconeogenesis from protein can defend these patients from hypoglycemia and why they demonstrate a normal hyperglucemic response to galactose and fructose infusions.

Information concerning the genetics of this condition are meager. Two of Cori's cases were in the same family, two of Hers' cases were brothers, another of his cases had a brother with glycogenosis and the parents of still another of his cases were first cousins. Again inheritance through a single recessive autosomal gene seems most probable.

Treatment of this disease with its milder manifestations is less difficult than is that of glucose 6-phosphatase deficiency although the same general principles apply. A more varied diet can be offered and a high protein content may be beneficial. It seems logical to avoid a diet high in fat because of the ketogenic potentialities. No reports of the effects of L-thyroxine therapy or sustained glucagon therapy in this condition have as yet appeared but one might expect that substantial glycogen mobilization from the liver via glycolysis could not be effected because of the inability to depolymerize proximal to the first branch point. It is possible however that glucagon therapy might allow a greater proportion of the alimentary influx of glucose to reach the peripheral blood.

Because of the probability of myocardial involvement Cori advises prohibition of strenuous exercise and Hers cautions about the use of anesthetics and cardio-depressant drugs.

#### BRANCHER DEFICIENCY AMYLOPECTINOSIS

**Synonyms:** Andersen's disease, type 4 of Cori, familial cirrhosis of the liver with storage of abnormal glycogen.

This is either a very rare disease or a very difficult one to recognize; only a single example has so far come to light. The patient observed and studied by Andersen [91] was an 11½ month old boy whose brother had died at the age of 7 months with the autopsy diagnosis of von Gierke's disease. The child had an enlarged liver and spleen and was icteric. Fasting blood sugars ranged between 56 and 75 mg per 100 ml and there was a diminished hyperglycemic response to epinephrine. The results of liver function tests were strikingly abnormal. Subsequently the child developed ascites and deepening icterus and died at the age of 17 months.

Autopsy revealed glycogen infiltration of liver, spleen, lymph nodes and intestinal mucosa. Nodular cirrhosis of the liver was also present. Glycogen in small amounts was present in the muscle fibers of the diaphragm but not in the kidneys.

accumulation infiltration with fine granules of glycogen and a definite increase in periportal connective tissue

The functional tests which should allow presumptive clinical differentiation of this disease and the typical expected results are

- 1 Hypoglycemia with fasting
- 2 Diminished hyperglycemic response to epinephrine or glucagon depending on the amount of time since the last feeding. Near normal responses are more likely in this disease because of the ability to mobilize the external chain glucose moieties if they are present as a result of a recent feeding
- 3 Normal hyperglycemic response to infusions of galactose and fructose provided an advanced cirrhosis is not also present

As is the case with all glycogenoses the diagnosis can be made with certainty only from the biochemical analysis of biopsy material. The confirmatory biochemical findings first described by Illingworth and the Coris [31] are

- 1 Increased liver and muscle glycogen content with the latter in the 4 to 5 per cent range or higher
- 2 Abnormally structured glycogen in both liver and muscle with short external chains if obtained during fasting and an increased number of branch points (10 to 13 per cent versus 7 to 8 per cent in the normal) under any conditions. Thus the molecular structure is very similar to  $\text{LD}_1$  (phosphorylase) or normal glycogen hence Coris' name "limit dextrinosis"

- 3 Absence of detectable amylo-1,6-glucosidase activity with relatively normal amounts of phosphorylase and glucose 6-phosphatase

Amylo-1,6-glucosidase in contrast to glucose-6-phosphatase is normally present in numerous tissues. This accounts for glycogen deposition in skeletal and cardiac muscle when the enzyme is lacking. The cardiac involvement is usually not recognizable clinically. Normal electrocardiographic findings must not be taken as proof that such involvement and its special hazards do not exist.

Recognition of the extent and nature of the enzymatic lesion in this disease allows a better understanding of its pathophysiology. Glycogen synthesis proceeds normally when glucose is in good supply and the arborized molecule is formed. With glucose deprivation or stimuli for glycogenolysis only the external branches of the molecule can be depolymerized by phosphorylase. When the first tier of branch points is reached the process stops because the specific enzyme necessary for cleavage of the 1,6 bonds is lacking. With return of sufficient glucose to allow storage or withdrawal of glycogenolytic stimuli extension of the 1,4 linked external chains recommences and when they are sufficiently elongated they are acted upon by the brancher enzyme to create a new 1,6 linked branch point. This new 1,6 bond effectively traps the glucose

toms disappeared rapidly upon resting the muscle but tended to persist longer if he attempted to continue the use of the muscle. The amount of work which produced pain varied from day to day and seemed to be less when he had infection. There was no history of a similar disturbance in his siblings, parents, or progeny.

Physical examination showed that he was well proportioned although he had a moderate dorsal kyphosis. There were no stigmas of any of the growth dystrophies or evidence of gross intellectual deficit. The size, initial power, and tone of all muscles were normal and no fasciculation or fibrillation could be detected nor could myoclonic phenomena be elicited. Results of the neurologic examination were normal.

Radiologic and hematologic findings and serum electrolytes including calcium and magnesium were all within normal limits.

Findings on functional tests were as follows:

1. Marked tachycardia after climbing if exercise was continued to the point of muscle weakness and ankle clonus.

2. Under ischemic conditions only 10 to 20 per cent of the normal amount of work could be performed by the forearm and hand muscles.

3. Maintenance of the contracted state in the exhausted muscle gave the appearance of localized swelling.

4. Complete absence of electromyographic activity which had been normal at the outset of exercise in the exhausted contracted muscle.

5. Following ischemic muscle work to the point of exhaustion there was a much greater reactive increase in blood flow than occurred in normal persons under the same conditions.

6. Fall in concentration of lactate and pyruvate in the venous return from an ischemic muscle performing work in marked contrast to the brisk rises of both these acids under similar conditions in a normal individual.

7. A normal hyperglycemic response to epinephrine but a smaller rise in blood lactate than produced in a normal person with a similar rise in blood sugar.

The information from the cases reported by Pearson [136] and Schmid [137] confirms that the inability to detect an increase in blood lactate after exercise especially if performed under ischemic conditions is the best indication obtainable by functional testing that myophosphorylase deficiency is present. Such a test should be performed in any investigation of muscular weakness developing after unusually short periods of exercise. Pearson found that exercise tolerance in his case could be markedly improved by continuous intravenous infusion of either glucose, fructose, or lactate but not by galactose, glycerol, or fat.

Histologically excessive amounts of glycogen in a diffuse granular form have been found in the muscle. Unequivocal diagnosis again depends on studies of biopsy material.

Structure analysis of the glycogen obtained from this case was carried out by Illingworth and Cori [134] and revealed a molecule with a structure quite similar to that of the amylopectins of plant starch. Fifty per cent of the molecule could be degraded by phosphorylase implying very long external chains. There were fewer branch points and the inner chains were longer than in normal glycogen. The physical properties of this abnormal molecule also resembled those of amylopectin in that it gave a purple iodine color with an absorption peak at 530  $m\mu$  while normal glycogen has a peak at 470 and corn amylopectin at 550. Normal glycogen is very soluble in cold water but this material was only sparingly soluble and reprecipitated at 0°C as does amylopectin.

Cori postulated that these structural abnormalities could be explained by a decreased activity of the brancher enzyme amylo-(1,4  $\rightarrow$  1,6) transglucosidase but unfortunately not enough tissue was available for the direct enzymatic assay necessary for confirmation. Recant speculated that the cirrhosis might be a tissue reaction provoked by the precipitation of the relatively insoluble polysaccharide [63]. Since this abnormal glycogen molecule should be subject to the usual reactions of degradation Cori suggested that its precipitation with attendant local tissue reaction made it inaccessible to phosphorylase and that this accounted for its deposition in increased amounts.

More study of cases of this type of defect will have to be carried out before information concerning genetics and treatment can be obtained. In searching for further examples it might be well to remember Andersen's description—Familial Cirrhosis of the Liver with Storage of Abnormal Glycogen.

#### MYOPHOSPHORYLASE DEFICIENCY GLYCOGENOSIS

Synonyms McArdle's syndrome McArdle-Schmid Pearson disease type 5 of Cori

In all at least eight patients with similar clinical and laboratory findings characteristic of this disease have been observed. Tissue from two of them has been subjected to exhaustive biochemical studies [135-137] which have elucidated a single enzyme deficiency lack of muscle phosphorylase as the cause of the condition.

Although McArdle [138] did not have the advantage of biochemical studies of excised tissue and interpreted the results of his physiologic studies as indicating a lesion of muscle glyceraldehyde phosphate dehydrogenase it seems almost certain that his patient had this disease. His clinical description is the most complete presently to be found in the literature and will be used to characterize the clinical features.

The patient was a 30 year old man who for as long as he could remember had experienced first pain and then weakness and stiffness with variable periods of exercise of any muscle including the masseter. Sym-

intact chain of associated enzymes along a given metabolic pathway strongly suggests that the defect is a result of a single genetic determination. There is no difficulty in understanding the mechanisms by which characteristic clinical symptoms and signs are produced during exercise since there is a biochemical lesion which prohibits the release of reserve fuel. The anomalous fall in lactate with muscle work probably indicates that it is being used to make up the fuel deficit at the same time that its production is being curtailed.

Beyond advice to avoid exercise to the extent of producing symptoms there seems little to offer the patients therapeutically. Perhaps the constant use of glucose lozenges during periods of necessary activity might prolong the duration of the symptom-free phase. Avoidance of tight garters, cuffs, collars and girdles would seem to be indicated also.

#### HEPATOPHOSPHORYLASE DEFICIENCY GLYCOGENOSIS

Synonyms: Hers disease type 6 of Cori

In his investigation of 15 cases of glycogenosis Hers found three apparently unrelated persons with an approximately 75 per cent reduction in assayable liver phosphorylase [8]. In two of the cases the liver glycogen content was over 12 per cent; in the third case despite a glycogen content of only 2.2 per cent assurance is given that the clinical and histopathologic criteria for a glycogenosis were fulfilled. Assay of glucose 6-phosphatase, amylo 1,6 glucosidase in liver and muscle and muscle phosphorylase gave near normal values. Muscle glycogen content was normal.

No clinical description of the cases has as yet been provided but it might be guessed that they resemble patients with glucose 6-phosphatase deficiency disease or debrancher deficiency limit dextrinosis with fasting hypoglycemia and subnormal hyperglycemic responses to glucagon. As in limit dextrinosis the galactose or fructose infusion test results in a marked hyperglucemia.

The intrinsic nature of this phosphorylase defect had not been completely elucidated at the time of the report but the presence of some phosphorylase activity apparently led the author to suspect a primary defect in either the dephosphophosphorylase kinase or the phosphorylase-phosphatase systems even though additions of ATP,  $Mg^{++}$  and adenosine 3,5-monophosphate failed to increase activity. It was also pointed out that since phosphorylase is the rate-limiting step in glycogenolysis and since its activity in the phosphorylytic direction is from 2.5 to 5.5 weaker than in the synthetic direction a 75 per cent reduction of activity (assayed under optimal conditions in the synthetic direction) would account for the excessive glycogen deposition. The normality of the glycogen content and phosphorylase activity found in the muscles is another indication among other evidences that myophosphorylase

Such biochemical studies on excised muscle from patients with this disease have shown the following:

1 Increased deposition of glycogen (2.5 to 4.1 per cent *versus* 0.2 to 0.9 per cent normally)

2 Inability of homogenates of biopsy tissue to form lactate *in vitro* from endogenous glycogen or added normal human glycogen, but an intact glycolytic capacity as indicated by normal lactate production after addition of glucose 1 phosphate

3 Normally structured glycogen by analytic enzymatic degradation [59]

4 Essentially undetectable phosphorylase *a* and *b* activity despite additions of AMP or pyridoxal 5-phosphate. Additions of crystalline phosphorylase to homogenate show no loss in activity

5 Adequate activity of the enzymes concerned with phosphorylase *a* and *b* interconversion, i.e., PR enzyme and phosphorylase kinase. The other enzymes concerned with glycogen synthesis and breakdown can also be detected as functionally adequate i.e., phosphoglucomutase, amylo-1,6-glucosidase, UDPG glycogen transglucosylase, and UDPG pyrophosphorylase [50, 59, 137]

Since excessive glycogen deposition occurs in the muscles in this disease and there is absence of phosphorylase synthesis must occur via the UDPG glycogen transglucosylase pathway, and this fact has been previously presented as part of the evidence for partition of glycogen synthesis and breakdown. Why greater accumulation of glycogen such as is seen in idiopathic generalized glycogenosis does not occur in muscles without phosphorylase remains a mystery. Whether cardiac muscle is also involved in myophosphorylase deficiency remains to be answered when studies of autopsy material can be made. Since cardiac muscle habitually uses substrates other than glucose as the major fuels of its energy production involvement with this defect might not be detectable or incapacitating. No interference with mechanical action due to the moderate glycogen deposition is detectable in the rested skeletal muscle and a similar degree of glycogenosis of cardiac muscle need not interfere with its contractile capacity.

It is clear from the normal results obtained with glucagon tests that hepatic phosphorylase activity is intact in this disease. This fact is not surprising when it is recalled that the two enzyme proteins are immunologically distinct and have different mechanisms of activation and deactivation. It seems highly probable therefore that there is a separate gene for each of the two phosphorylases if this disease is genetically determined. Insufficient information is available concerning sibling occurrence, sex distribution, consanguinity of parents, and familial occurrence to reach a conclusion regarding the mode of inheritance of this defect. However, the sharp specific absolute absence of a single enzyme in an otherwise

intense interest to biochemists since the time of Claude Bernard and have been worked out with considerable clarity

■ Humoral and other regulatory factors can influence the ebb and flow of glycogen metabolism. The mechanisms by which such agents act are in some cases obscure and complex but much information concerning their actions is already available. Certain tissues appear to have specialized systems in regard to the glycogen cycle. This fact makes it necessary to characterize the glycogen metabolism of any given tissue independently.

3 Clinical examples of excessive glycogen deposition in several or all organs and tissues have been observed. Five of the six currently recognized diseases appear to be biochemical lesions due to a genetically determined absence or deficiency of a single enzyme concerned with the degradation or structural modification of stored glycogen. In each of these syndromes the mode of transmission seems to be as a simple autosomal recessive gene. A form of generalized glycogenosis is known in which as yet no enzymatic or biochemical lesion has been elucidated.

4 The steps of the glycogen cycle and the enzymes involved appear in schematic form in Fig 6-12. The point of blockage and the genetically determined enzyme deficiency are indicated along with the suggested clinical name for each syndrome.

5 Consideration of the position of each lesion in the framework of the knowledge concerning the steps of the glycogen cycle has made possible understanding of many of the clinical findings as well as proper interpretation of functional diagnostic tests. Definitive diagnosis cannot be made from clinical and functional test information with absolute certainty. Direct investigation of the activities of the various pertinent enzymes in the involved tissues must be made in order to characterize unequivocally any given example of a glycogen deposition disease.

6 At the present therapy in these diseases is defensive and supportive. Better understanding of the biochemistry involved has allowed employment of logical measures which in many instances are gratifyingly effective.

7 It now appears that a human disease state has been found for the isolated deficiency of every currently described enzyme whose absence could conceivably lead to excessive glycogen deposition. Nevertheless as astute clinicians continue to scrutinize their cases they will provide undoubtedly problems, questions and clues along with tissue for study by their biochemist allies and diseases of greater subtlety and complexity will be recognized and understood.

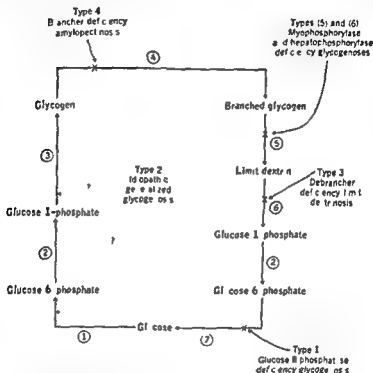
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and hepatic phosphorylase are different enzyme proteins which may have separate genetic determinants.

Until more is known concerning the essential nature of this defect and more cases are recognized and studied as to genealogy, no statement can be made concerning its mode of inheritance or methods of therapy.



#### Enzymes

- (1) Hexokinase (glucokinase)
- (2) Phosphoglucomutase
- (3) UDPG:glycogen transglucosylase
- (4) Amylo (1,4 → 1,6) transglucosidase
- (5) Phosphorylase
- (6) Amylo 1,6-glucosidase
- (7) Glucose 6-phosphatase

Fig. 6-1 The glycogen cycle (chart) and names of genetically determined enzyme lesions causing excessive glycogen deposition.

## SUMMARY

1. Glycogen is a polydisperse polysaccharide of high molecular weight present in almost all tissues of the animal body. It occupies a role in the economy of metabolism as a storage carbohydrate similar to the role of the starches and amylopectins in the plant kingdom. The enzymatic steps involved in its synthesis and degradation have been the subject of

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milk ingestion develop vomiting and diarrhea which are usually followed by dehydration. The subsequent poor caloric intake and gastrointestinal losses contribute to impaired nutrition and growth. Jaundice is a common occurrence and reflects the parenchymal liver involvement that often accompanies the disease. Initially the liver shows fatty infiltration which

TABLE 7-1 CLINICAL AND LABORATORY FINDINGS IN GALACTOSEMIA

*Clinical Features**Laboratory Findings*

- |                               |  |
|-------------------------------|--|
| 1 Nutritional failure         | 1 Elevated blood galactose                                     |
| Hepatosplenomegaly, cirrhosis | 2 Abnormal galactose tolerance                                 |
| 3 Cataracts                   | 3 Diluent erythrocyte galactose-1-phosphate uridyl transferase |
| 4 Mental retardation          | 4 Urine galactose, amino acids, albumin                        |

readily proceeds to cirrhosis if the milk ingestion persists. In some cases the disease is fulminating and death may occur in the neonatal period or soon thereafter. If the clinical manifestations extend beyond 4 to 8 weeks one can usually detect evidence of cataract formation followed subsequently by mental retardation. These infants are usually reluctant to eat and may show lethargy and hypotonia. Depression of blood glucose levels during milk ingestion is not unusual and symptoms typical of hypoglycemia may occur. There may exist therefore an elevated blood galactose level together with a low blood glucose. This may also occur during a galactose tolerance test (Fig 7-1). The experimental observations of Fox [4] suggest that the hypoglycemia may be due to stimulation of insulin release from the pancreas by the elevated blood galactose.

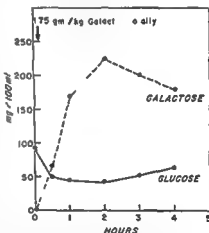


Fig 7-1 Effect of an oral galactose tolerance test on blood glucose and galactose levels in an 8-year-old patient with galactosemia.

It should be emphasized that in addition to cases of galactosemia with the classic features as described above, there are other patients with less pronounced manifestations in whom disturbances of growth, liver function, or the central nervous system may not attract attention for weeks or months. Frequently these patients are ones who have shown milk intolerance in the early days of life. Presumably because of reduced milk intake and the use of milk substitutes they have not developed the severe clinical manifestations. It is readily apparent how the diagnosis may be missed in such patients on clinical grounds alone. Patients



## Chapter 7

### Galactosemia\*

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Kurt J. Isselbacher

The disease galactosemia is now well recognized as a congenital and hereditary error in the metabolism of galactose. Patients afflicted with this disorder have an impairment in their ability to utilize ingested galactose and to convert it to glucose or energy. The clinical manifestations are directly related to galactose administration and the disease may be fatal if galactose ingestion continues. In contrast, upon removal of the offending carbohydrates from the diet most of the symptoms regress or even disappear completely. More than forty cases have been reported and are known to exist at the present time [1-3] and many more unreported cases have been discovered in the course of investigation of this disease.

It is the purpose of this chapter to discuss the clinical aspects of the disease and current methods available for its diagnosis and treatment. The biochemical reactions important in galactose metabolism and, specifically, the basic enzymatic defect in galactosemia are reviewed in detail. The current concepts of the genetic mode of transmission of the disease and the techniques available for the detection of the heterozygous state are also described.

#### CLINICAL ASPECTS

The symptoms which occur in galactosemia usually manifest themselves shortly after birth and are known to be causally related to the ingestion of galactose as found in the lactose of milk. The main clinical features in the classic cases include the triad of cataract formation, hepato-splenomegaly, and mental retardation (Table 7-1). The affected infants usually appear normal at birth but after some days or weeks of

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completely [2] The only major finding in which there usually is no improvement is the mental retardation It has been observed by most authors that the central nervous system damage may not improve despite the most rigid adherence to a galactose-free diet It is for this reason that early diagnosis and prompt institution of therapy are so important

Galactose free diets can be instituted in infancy by resorting to any number of commercially available milk substitutes such as Nutramigen Dextrin Maltose or soy bean preparations It has been amply demonstrated that these diets are compatible with normal development and growth even though as Holzel et al [11] point out they may still contain traces of galactose Most of the patients whom the author has maintained on these commercial preparations have done well In none have mental retardation cataract formation liver disease or any symptoms occurred as a result of these diets The author therefore does not share the view of Holzel et al that the traces of galactose in these commercial milk substitutes are harmful and that more strict artificial diets should be instituted On the contrary there is the consideration that the gastrointestinal disturbances that accompany the lactose-free diet of Holzel Komrower and Schwarz [12] may occasionally be a drawback in the very young infant

Although Platt [13] feels that galactose ingestion may be of special benefit and that galactose is poorly synthesized in the body there is at present no definite clinical evidence that central nervous system function or somatic development in general is impaired by the prolonged use of galactose free diets There is ample evidence as indicated below that patients with galactosemia possess the enzyme uridine diphosphate galactose 4 epimerase which allows for endogenous synthesis of galactose from glucose and hence permits galactolipid formation in the central nervous system [14-16] and elsewhere in the absence of dietary galactose

Galactosemia is now known to be due to the congenital absence of a specific enzyme galactose 1 phosphate uridylyl transferase [16-17] which accounts for the impaired galactose metabolism in this disease Nevertheless it has been frequently observed that patients with galactosemia may in later years of life ingest varying quantities of galactose without experiencing any significant side effects [1-3] This phenomenon is probably related to two factors (1) an actual improvement in the galactose utilization in these patients with increasing age and (2) the fact that in later years the ratio of ingested galactose to body weight or surface area is far smaller than in the neonatal period when the symptoms are so pronounced

The improvement in galactose tolerance with age at first seems paradoxical since even the adult has been shown to have a persistence of the enzymatic defect One explanation is that there is an additional enzyme permitting galactose utilization (see below UDI galactose pyrophosphorylase) which in the normal person is quite deficient at birth but

in mental institutions who have recently been discovered by means of the red cell enzyme test (see below) [5] to have galactosemia probably fall into this group

The chief clue to the diagnosis of galactosemia is usually the finding of a reducing sugar in the urine which when analyzed (see below) is identified as galactose. It is important to emphasize that the galactosuria may be inconstant for it is obviously dependent on galactose or lactose ingestion and this may be minimal if the patient has been vomiting. Errors of diagnosis have often been made when the patient was placed on parenteral fluid therapy upon admission to the hospital and the reducing sugar shown by initial urinalysis was mistakenly assumed to be due to the intravenous glucose.

In addition to galactosuria these patients may have variable degrees of albuminuria and aminoaciduria [6-8]. The amino acid pattern has been similar in most cases. There is a predominance of the neutral simple aliphatic chain type i.e. serine, glycine, alanine, threonine, glutamine and valine. In addition small quantities of phenylalanine, lysine, cystine, glutamic acid, methyl histidine, tyrosine and aminoisobutyric acids have been detected [6, 7]. It has been shown by Komrower [9] as well as by Cusworth, Dent and Flynn [10] that this aminoaciduria is primarily renal in origin. It occurs only in relation to galactose ingestion and galactosuria and is not observed in patients on galactose-free diets. In a study by Cusworth et al. [10] the urinary amino acid excretion was evident for as long as 7 days after the cessation of galactosuria. It is assumed that galactose or one of its metabolites interferes with amino acid reabsorption by the renal tubule. The fact that the aminoaciduria occurs only after several days of galactosuria [10] and persists for some days after galactose has disappeared from the urine is perhaps more in favor of the exertion of an inhibitory effect on the renal tubule by a metabolite of galactose. Galactose 1 phosphate which is known to accumulate in the tissues of galactosemic patients (see below) may be the metabolite which inhibits the renal tubular reabsorption of amino acids.

## NUTRITIONAL THERAPY AND SUBSEQUENT COURSE

If significant milk ingestion persists in patients with galactosemia death usually results. On the other hand if the diagnosis is made before the disease is too far advanced one of the striking features is that all the symptoms and signs may regress and even disappear completely if the patients are given galactose-free diets. Nausea, vomiting and diarrhea cease and there is rapid weight gain. The liver and spleen return to normal size. Cirrhosis if present has been known to disappear completely [1]. The urinary abnormalities mentioned above likewise promptly cease. Cataracts if present often regress and in some instances disappear

phogalactose (UDP galactose) while at the same time glucose 1 phosphate is liberated from the original nucleotide

Galactose 1 phosphate + UDP glucose  $\rightleftharpoons$  UDP galactose + glucose 1 phosphate (2)

The enzyme has been variously referred to as Gal 1 P uridyl transferase P Gal transferase or Gal 1 P transuridyase. This enzyme is of prime importance for the utilization of galactose 1 phosphate and its incorporation into UDP galactose.

It should be noted that another reaction (5) (see below) can also serve to incorporate galactose 1 phosphate into UDP galactose but does so to a lesser extent. UDP galactose is necessary for the synthesis of galactose derivatives such as lactose, galactolipids, and galactose-containing polysaccharides (e.g. chondroitin sulfate). That Gal 1 P uridyl transferase represents the main pathway in galactose utilization will be evident from the fact that this enzyme as described below is blocked or deficient in galactosemia. The enzyme has now been purified from liver [22] and can be used as a specific tool in the microdetermination of galactose 1 phosphate.

#### UDP galactose 4 epimerase (III)

In a third reaction the two uridine nucleotides are directly interconverted



The enzyme catalyzing this reaction was previously called  $\alpha$ -Waldenase but with more recent data showing the requirement for diphosphopyridine nucleotide (DPN) [23] it is more appropriately called an epimerase. Although definitive proof still needs to be obtained it appears that an epimerization occurs at carbon-4 of the hexose presumably by an oxidation-reduction reaction. The hypothetical 4-keto hexose intermediate has not been isolated but it seems highly probable that DPN functions here in the capacity of a hydrogen transporting agent.

It is important to note that this key enzyme catalyzes a reversible reaction and that therefore UDP galactose can be formed from UDP glucose. The latter is in turn derived from glucose 1 phosphate by the next reaction to be described (4). It is therefore evident that dietary galactose is not essential for UDP galactose synthesis and that the synthesis of such compounds as galactolipids may proceed from glucose via the epimerase despite the absence of Gal 1 P uridyl transferase.

#### UDP-glucose Pyrophosphorylase (IV)

This enzyme is not directly involved in the galactose-glucose interconversion but is a means of synthesizing the important compound UDP glucose involved in the preceding two reactions. The reaction is

which increases in activity with age [19]. The development and presence of this pyrophosphorylating system in spite of the persistent deficiency of the transferase enzyme, would readily explain the improved galactose metabolism in the galactosemic person as he matures.

## BIOCHEMICAL CONSIDERATIONS IN THE GALACTOSE-GLUCOSE INTERCONVERSION

Considerable knowledge has been gained in recent years concerning

the metabolism of galactose and its utilization in the body. Most of the ingested galactose is in the form of lactose the main carbohydrate of milk which in the intestine is split into its two component monosaccharides, galactose and glucose (Fig. 7.2). The sugars differ only in the orientation of the hydrogen and hydroxyl groups about the fourth carbon atom. The exact manner in which galactose is converted to glucose or glucose derivatives has been greatly elucidated in recent years principally as the result of investigations by Leloir, Kilekar and their associates [19-21]. The following enzymes and the reactions they

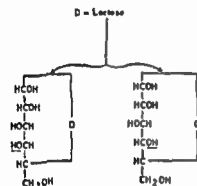


Fig. 7.2 Comparison of the chemical structure of galactose and glucose.

catalyze are important and deserve consideration in the galactose-glucose interconversions.

### Galactokinase (I)

This enzyme catalyzes the conversion of galactose to  $\alpha$ -galactose-1-phosphate. This requires adenosine triphosphate (ATP) in a reaction analogous to that catalyzed by hexokinase.



The reaction is not reversible and adenosine diphosphate (ADP) is also formed. The enzyme is known to exist in liver, brain, and red cells.

### Galactose-1-phosphate Uridyl Transferase (II)

The conversion of galactose-1-phosphate to glucose-1-phosphate is now known to occur by a reaction which involves a specific nucleotide, uridine diphosphoglucose (UDP-glucose). In this reaction the galactose-1-phosphate is transferred to the nucleotide to yield uridine diphos-

## ENZYMATIC DEFECT IN GALACTOSEMIA

In studies aimed at elucidating the possible cause of galactosemia it seemed reasonable to assume that the defect might involve one or more of the above enzyme reactions. Although the liver is the major site of the galactose-glucose interconversion it was shown by Schwarz et al [20] that the red cells also metabolize galactose and that galactosemic red cells are abnormal in this respect. These workers made the important observation that erythrocytes of galactosemic persons show an accumulation of galactose 1 phosphate after the ingestion of milk or galactose. These observations have been confirmed with *in vitro* experiments in which galactosemic red cells incubated in a galactose-containing medium showed significant amounts of galactose 1 phosphate in contrast to normal red cells similarly incubated [17]. Both these observations strongly suggested that the defect in galactosemia could be due to a block in the conversion of galactose 1 phosphate to glucose 1 phosphate and that such a block might reasonably involve the enzyme Gal 1 P uridyl transferase. The occurrence of UDP galactose pyrophosphorylase in mammalian tissues was not known until recently but since it does not seem to exist in red cells knowledge of it would not have precluded focusing attention on the deficiency of Gal 1 P uridyl transferase as a plausible explanation for the *in vivo* and *in vitro* accumulations of galactose 1 phosphate.

Biochemical studies of human erythrocytes aimed at elucidating the site of the abnormal galactose metabolism disclosed that these cells contain enzymes catalyzing Reactions 1 through 4. Galactosemic red cells were shown specifically to be deficient in enzyme II Gal 1 P uridyl transferase [17] (Table 7-2). This observation has been confirmed in all

TABLE 7-2 ENZYME ACTIVITY IN HEMOLYSATES OF NORMAL AND GALACTOSEMIC INDIVIDUALS

| Subjects     | Galactokinase |          | Gal 1 P uridyl transferase |          | UDP galactose 4 phosphate |          |
|--------------|---------------|----------|----------------------------|----------|---------------------------|----------|
|              | %             | Activity | %                          | Activity | %                         | Activity |
| Normal       | 3             | 0.10     | 15                         | 0.8      | 3                         | 0.3      |
| Galactosemic | 3             | 0.09     | 10                         | <0.0     | 3                         | 0.35     |

Activity (average) is given in micromoles of reactants converted per milliliter of erythrocytes per hour.

SOURCE: H. J. Iselbacher et al. Science 123:635, 1956.

the cases of galactosemia studied thus far. It was shown that galactosemic red cells did not lack any cofactors nor did they contain any demonstrable inhibitors to account for the lack of transferase activity. It was initially

reversible and requires uridine triphosphate (UTP). Pyrophosphate is formed as product of the reaction



This enzyme is abundant in liver, red blood cells, brain, mammary gland, and muscle.

#### UDP-galactose 4-epimerase (V)

Recent observations [25] indicate that mammalian liver also contains a pyrophosphorylase which catalyzes the following reaction



The activity of this enzyme in neonatal liver tissue is very weak, but it has been found to increase with age. Its activity (per milligram of protein)

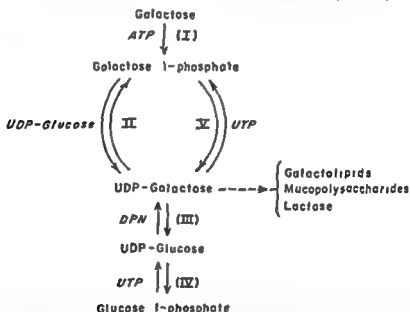


Fig. 7.3. A schematic representation of the reactions and interactions important in the metabolism of galactose. Roman numerals are the same as those in the text and refer to the specific enzymes catalyzing the various reactions. Abbreviations include: ATP, adenosine triphosphate; UDP-glucose, uridine diphosphate glucose; UDP, uridine diphosphate; galactose, galactose; UTP, uridine triphosphate. (From A. J. Isselbacher, *Am. J. Med.* 26: 715, 1959.)

in adult liver is about one-sixth that of Gal 1-P uridylyl transferase [18]. It is apparent that this enzyme provides an additional pathway by which galactose 1-phosphate may be utilized and incorporated into UDP-galactose. The enzyme is found in liver and brain but has not been detected in erythrocytes.

The interrelationships of these reactions are shown in Fig. 7.3.

From previous observations [17-25] one would expect that some of the retained galactose would be in the form of galactose 1 phosphate. Studies performed with  $C^{14}$  labeled galactose in a 24 year old male with galactosemia have permitted a more definitive analysis of this problem [27]. The results are indicated in Table 7-4. It is observed that 3 per cent of the administered galactose could be accounted for in the urine as a glucosiduronic acid. In view of present knowledge of the mechanism of glucosiduronic acid formation the data indicate that at least 3 per cent of the administered galactose must have been converted to glucose derivatives (via the uridine nucleotides). These observations suggest either that the deficiency of Gal 1 P uridyl transferase is incomplete in this disease or that there exists alternate or accessory pathway for galactose metabolism. Since the enzymatic deficiency in the red cells is virtually complete the possibility of other routes of galactose utilization by passing the transferase reaction must be considered. It is evident that

TABLE 7-4 METABOLISM OF  $C^{14}$  GALACTOSE BY AN ADULT PATIENT WITH GALACTOSEMIA

| Determination   | Amount mg |
|---|-----------|
| Galactose in urine  | 700       |
| Galactose metabolized to glucosiduronic acid                              | 30        |
| Galactose accumulating as cellular galactose (galactose 1 phosphate etc.) | 50-100†   |

Values after infusion of 1 gm galactose and 5 microcuries galactose  $C^{14}$  to a 4 year-old male with galactosemia. 1 gm menthol was given orally.

† All tissues with the exception of muscle and bone.

SOURCE: F. Eisenberg Jr. et al. Science 125:116, 1957.

the enzyme UDP galactose pyrophosphorylase could function in such a manner and allow for galactose 1 phosphate utilization and incorporation into UDP galactose. This theory is appealing because this enzyme has a very low activity in fetal and neonatal tissue but increases in activity with age [18]. It is reasonable therefore to postulate that patients with galactosemia have their most pronounced symptoms at birth because at this time they have both an absent transferase system and low UDP galactose pyrophosphorylase activity. The subsequent increase in activity of the latter enzyme with age would then explain the improved tolerance and galactose utilization which occurs as the galactosemic patient matures.

## DIAGNOSTIC TESTS

### CHARACTERIZATION OF THE NATURE OF THE MELITURIA

When one finds a reducing sugar in an infant with or without the classic features of galactosemia described above it is imperative to establish



reasoned that the absence of the enzyme might simply be due to an adaptive phenomenon i.e. since many of the galactosemic patients tested had been off galactose for extended periods of time the red cells might gradually have lost their transferase activity. However, when erythrocytes of patients with milk allergy who had been off galactose for as long as 18 months were examined they were found to be normal with respect to all of the three other enzymes (I-III-V). Also it has been shown that the transferase deficiency exists at birth by demonstrating the absence of the transferase in the cord blood of infants afflicted with the disease [26].

The enzymatic defect noted in the erythrocytes has also been investigated in the liver tissue of two afflicted patients [26]. In order to estimate the activity of the enzyme in samples of liver tissue (biopsies) a different technique was necessary. Carbon 14 labeled galactose 1 phosphate was used and the rate of incorporation of radioactivity into the uridine nucleotide fraction was measured. This method is more sensitive than the spectrophotometric one used in the erythrocyte studies and allows detection of enzyme activity of as little as 1 per cent of the normal. It will be seen in Table 7-3 that the infant liver contained no detectable activity while the adult showed a rate of incorporation of  $C^{14}$  galactose 1 phosphate which was 4 to 5 per cent of that in the nongalactosemic liver. It should be emphasized that this isotopic method is not so specific as the spectrophotometric one and would not distinguish between enzymes II and V. Therefore the small activity in the adult galactosemic liver may be due to the enzyme UDP galactose pyrophosphorylase (V) (see below).

TABLE 7-3 URIDYL TRANSFERASE ACTIVITY IN HUMAN LIVER HOMOGENATES

| Subject                              | $C^{14}$ labeled<br>Gal 1 P<br>Incorporated |
|--------------------------------------|---|
| Nongalactosemic adult (post mortem)  | >100  |
| Nongalactosemic infant (post mortem) | >200  |
| Galactosemic adult (biopsy)          | 12  |
| Galactosemic infant (biopsy)         | <0.1  |

Activity in micromoles of label incorporated per gram of liver per hour  
SOURCE: E. P. Anderson et al. Science 125:113, 1957

### POSSIBLE ALTERNATE PATHWAYS OF GALACTOSE METABOLISM IN GALACTOSEMIA

As indicated above, observations have been made by several groups of investigators which suggest that administered galactose can be metabolized to some degree in galactosemic patients [3]. These conclusions have been based on the fact that from 30 to 80 per cent of ingested galactose is retained and not accounted for by urinary excretion of the sugar

## GALACTOSE 1 PHOSPHATE ACCUMULATION IN ERYTHROCYTES

Schwarz, Holzel, and Komrower have advocated the use of an *in vitro* test based on the accumulation of galactose 1 phosphate in galactosemic red cells [32]. In this test the red cells are incubated with galactose 1 phosphorylated derivatives are then isolated as their barium salts. The sugar phosphates are hydrolyzed and the free sugars separated by paper chromatography. The galactose is estimated by comparison with known quantities chromatographed simultaneously. The main disadvantage of this test is that it requires 2 to 3 days to carry out. As with the enzymatic procedure, this test can be performed on cord blood and the diagnosis made several days after birth.

## POSSIBLE FACTORS IN TOXICITY

Although galactosemia has been observed only in man, somewhat analogous physiologic changes can be induced in animals by feeding diets high in galactose. It has been observed repeatedly that when chicks are placed on 30 per cent galactose diets they may show a quivering syndrome [33] and rats uniformly develop cataracts within a period of 14 to 21 days [34]. In the galactose-fed rats with cataracts, examination of the lenticular tissues [35] has revealed significant amounts of galactose 1 phosphate, just as in the galactosemic red cells. It is tempting to speculate that this hexose-phosphate accumulation may interfere with or inhibit one or more enzyme systems.

Experimental evidence to date bears out some of these speculations. Thus it has been recently reported [35, 36] that galactose 1 phosphate inhibits phosphoglucomutase, provided that glucose 1,6-diphosphate is not present in excess. The physiologic significance of this interference is at present difficult to evaluate, but it may be another factor contributing to the hypoglycemia which occurs in galactosemic patients when they ingest galactose or lactose.

Recently Lerman [36a] has indicated that there seems to be an inhibition of glucose 6-phosphate dehydrogenase in the lens of rats fed on high galactose diets. It was suggested that galactose 1 phosphate accumulation in the cataract was responsible for this inhibition. The author [36b] has studied the activity of purified glucose 6-phosphate dehydrogenase of red cells<sup>2</sup> and has found no inhibition at concentrations up to ten times that of the substrate, glucose 6-phosphate. It would seem therefore that the observations of Lerman do not seem valid for the glucose 6-phosphate dehydrogenase of tissues other than the lens.

The consumption of ATP in the phosphorylation of galactose to galactose 1 phosphate in these patients could conceivably also result in

<sup>2</sup> kindly supplied by Dr. Paul A. Marks.

the identity of the sugar. Recently this has been greatly facilitated by methods utilizing the enzyme glucose oxidase. Commercial test preparations containing glucose oxidase such as Tes-tape and Clinistix will give a positive reaction only in the presence of glucose<sup>1</sup> thus nonglucose sugars can readily be suspected. A recently reported method [29] using a microbial galactose oxidase is promising since it will permit rapid and definitive demonstration of galactose in biologic fluids. In addition to the usual chemical methods for identifying galactose (e.g., oxidation of the sugar to mucic acid) paper chromatography has been employed to assist in the diagnosis [30].

### GALACTOSE TOLERANCE TEST

In the past it has been customary to confirm the diagnosis of galactosemia by means of the oral or intravenous galactose tolerance test [1-3]. This test is not without hazard especially in an infant in whom there is marked impairment of galactose utilization. These patients often develop hypoglycemia during the course of the test (Fig. 7-1) and serious reactions such as convulsions may occur. For this reason and because infants with galactosemia should preferably not be exposed to galactose *the galactose tolerance test should be avoided if at all possible especially since one can now make the diagnosis by other tests cited below which do not jeopardize the health of the infant*.

### ERYTHROCYTE ASSAY FOR GAL-1-P URIDYL TRANSFERASE

As a result of the observations that circulating erythrocytes normally contain abundant amounts of Gal-1-P uridyl transferase a test has been developed which is based on the absence of this enzyme in galactosemia. This method the details of which have been outlined elsewhere [31] is both sensitive and specific. No false positives have been encountered. Patients with diseases such as hepatitis or cirrhosis in whom galactose tolerance is abnormal do not demonstrate a defect of transferase activity in their red cells. Errors may occur if a galactosemic patient has had a transfusion of normal blood within a period 3 months prior to the assay. In such a situation the transferase content of the transfused normal cells may give a false result [26]. This enzymatic method has a number of advantages: (1) it is quickly performed and the result can be obtained in several hours; (2) it permits diagnosis at birth on umbilical cord blood a feature desirable if the newborn infant is a member of a galactosemic family; (3) it avoids the hazards of galactose administration; (4) it is specific.

Some purified fungal preparations of glucose oxidase have been reported to oxidize galactose to a small extent [28]. This does not seem to be a problem with the enzyme used in Tes-tape and Clinistix.

A method much more suitable for genetic studies has recently been developed by Kirkman and Bynum [41]. Their assay is a manometric one using hemolyzates of human erythrocytes and is carried out in such a manner that zero-order kinetics obtain. In a study of 18 parents of galactosemic patients, all parents possessed enzymatic activity below the normal mean. Thirteen of the 18 parents had activities which were more than two standard deviations below the normal mean. Some of their data are reproduced in Fig. 7-4. It is apparent that parents, as well as normal

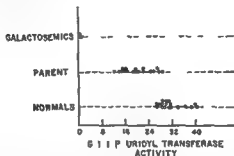


Fig. 7-4. A comparison of the galactose 1 phosphate uridyl transferase activities in galactosemic patients, their parents, and normal controls. Activity is expressed as microliters of oxygen uptake per hour for 0.3 ml. Represents a galactosemic patient after transfusion with normal blood. (By permission of H. V. A. Laman et al. [41].)

persons vary somewhat in the amount of Gal 1 P uridyl transferase activity, and parents and the normal controls are seen to overlap slightly in their upper and lower extremes. It is possible, as these authors point out, that the variation in the observed enzymatic activity represents a combination of environmental factors, laboratory error, and possibly even other genes, but that nevertheless all parents are heterozygous and possess diminished transferase activity.

Recently Weinberg, Herring, Johnson, and Field [43] have employed the oxidation of galactose-1- $C^{14}$  to  $C^{14}O_2$  by leukocyte suspensions or whole blood to study patients with galactosemia. They have observed that there is only negligible production of  $C^{14}O_2$  by cells from galactosemic individuals when incubated with  $C^{14}$ -labeled galactose. With this sensitive technique they were able to detect and quantitate the carrier state in one family.

Using this method the author has investigated the problem of whether the enzymatic defect in galactosemia is complete or only partial. The spectrophotometric assay for galactose 1 phosphate uridyl transferase has been very suggestive of a complete defect, but the sensitivity of the assay is such that demonstration of the complete absence of the enzyme has not been possible.

The experiments performed were based on the observation that the oxidation of galactose by erythrocytes is markedly enhanced (ten to

cellular disturbances. Thus the cells would be yielding a significant amount of their ATP to form a product (galactose 1 phosphate) which accumulates and is not readily metabolized. Of relevance in this connection are observations by Pennington and Prinkerd [37] that the ATP content of a galactosemic patient's red cells decreased when the individual was placed on a lactose diet.

The hypoglycemia which may occur in this disease has also been considered by many as causally related to the clinical manifestations of the disease, especially in regard to the mental retardation. It should be recalled that the symptoms and signs found in galactosemia notably the lenticular and central nervous system changes do not occur in such conditions as von Cierke's disease where episodes of hypoglycemia are pronounced and severe.

### GENETIC CONSIDERATIONS

A number of the inborn diseases of metabolism have been shown to result from a combination of two recessive genes. Phenylpyruvate oligophrenia is a typical example of a disease transmitted as a single recessive trait: parents of the afflicted patients show a distinctly lowered tolerance for phenylalanine [38].

The frequent occurrence of galactosemia in siblings and among the offspring of consanguineous matings [11] together with its equal incidence in both sexes suggests that this disease is transmitted by a single autosomal recessive gene. Holzel and Komrower [39] have investigated the genetics of galactosemia using the oral galactose tolerance test. They found mild impairment of galactose tolerance in some relatives but were unable to show abnormal tolerance in both parents. It is difficult to reconcile this observation with the concept that galactosemia is a simple recessive disease in which both parents should be heterozygous. It is apparent that this test is not sufficiently sensitive to allow definitive conclusions concerning the genetic mode of inheritance in galactosemia. This same limitation applies to the use of the Gal 1-P uridyl transferase assay for genetic studies. Although this test is reliable and specific the kinetics of the method are such as to make it difficult to assay quantitatively intermediate amounts of the enzyme. Nevertheless Hsia et al [40] have used the transferase assay in genetic studies. In a preliminary report of their observations on five galactosemic families they described decreased Gal 1-P uridyl transferase activity in heterozygotes. It should be noted that the differences in values between their normal controls ( $4.5 \pm 0.47$  units per gm hemoglobin) and the heterozygotes ( $3.3 \pm 0.36$ ) make it difficult to interpret the results in any single individual. Kalchauer et al were unable to find any significant differences between parents and normal controls using the transferase assay [31].

2 The disease is transmitted by a single autosomal recessive gene and is expressed as a characteristic cellular deficiency of the enzyme galactose 1 phosphate uridyl transferase

3 The clinical manifestations consist of nutritional failure hepatosplenomegaly with cirrhosis cataracts and mental retardation The major laboratory findings are galactosuria aminoaciduria albuminuria and an impaired galactose tolerance test result All these manifestations may disappear or regress if galactose is removed from the diet

4 The cause and mechanism of the toxic manifestations are not clear They appear to be related to the accumulation in the tissues of galactose 1 phosphate

5 The presence of galactose 1 phosphate uridyl transferase in normal erythrocytes and its absence in galactosemic cells have provided the basis for rapid and specific diagnostic tests for this condition

6 Some improvement in galactose utilization occurs in these patients as they mature This may be related to the activity of an accessory pathway of galactose metabolism via the enzyme uridine diphosphogalactose pyrophosphorylase

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twentyfold) by an electron acceptor dye such as methylene blue at a concentration of  $10^{-4}M$ . The effect is comparable to that observed by Brin and Yonemoto [43] with glucose. In the presence of a partial deficiency of uridyl transferase one would expect some stimulation of galactose oxidation although perhaps diminished. However in the presence of a complete enzyme lack no enhanced oxidation should occur. It has been observed (Table 7.5) that the red cells from parents of galactosemic patients have a reduced stimulation with methylene blue (sixfold increase) while cells from the homozygous individuals show no response to the addition of dye. These findings strongly suggest that at least in the red cell the enzymatic defect is complete.

TABLE 7.5 THE OXIDATION OF GALACTOSE  $C^{14}$  TO  $CO_2$  AND THE EFFECT OF METHYLENE BLUE BY RED CELLS FROM NORMAL SUBJECTS AND IN HOMOZYGOTES AND HETEROZYGOTES FOR GALACTOSEMIA

| Subject                | $C^{14}O_2$ lib. at 1 hr per 100 mg Hb | Per cent stimulation of $C^{14}O_2$ oxidation by methylene blue $10^{-4}M$ |
|------------------------|--|--|
| Normal (5)             | 11,800                                 | 100  |
| R.D. Motter            | 5,264                                  | 53.9   |
| F.D. Fatter            | 9,740                                  | 57   |
| I.D. 1 yr galactosemic | 290                                    | 1  |
| C.D. 4 yr galactosemic | 0                                      | 3  |

The incubation system contained 0.5 ml washed red cells, 0.05 microcurie galactose  $C^{14}$  methylcellulose  $10^{-4}M$  and 2.5 buffer [44] to a total volume of 1.5 ml. Incubation was for 90 min at  $37^\circ C$ .

Most recently Bretthauer et al. [42] have successfully employed a modified method of assay of Gal 1 I uridyl transferase in which hemolyzed red cells are briefly incubated with optimal amounts of Gal 1 P and UDP-Glc. Segregation of galactosemic patients, presumed heterozygotes and normal subjects appeared to be complete.

It still is not known whether the heterozygous individuals have a reduced amount of unaltered and structurally normal enzyme or whether the lowered activity which they demonstrate is the result of some hybrid gene product. These and other considerations as they apply to biochemical genetics have been admirably reviewed by Kalkkar [45].

## SUMMARY

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bilirubin in biologic specimens [12] When icteric crums were studied with this method it soon was recognized that bilirubin appeared to be present in two different forms one gave a *direct* reaction when diazotized sulfanilic acid was added to icteric serum whereas the other coupled with the diazo reagent only after alcohol was also added to the reaction mixture (*indirect* reaction) Separate determination of these two pigment fractions was found to be of clinical usefulness in the differential diagnosis of jaundice [13] For many years the basic reason for this difference in reaction type was unknown and numerous explanations and hypotheses were offered [14]

The discovery that bilirubin is excreted in conjugated form [15-17] provided a simple explanation for this problem Conjugated bilirubin is water soluble and reacts with diazotized sulfanilic acid in the absence of alcohol (*direct* van den Bergh reaction) whereas native bilirubin is insoluble in water [18] and requires addition of solvents to permit coupling with the diazo reagent (*indirect* van den Bergh reaction)

The finding that for excretion in the bile bilirubin is converted to water soluble conjugates throws new light on the way by which the pigment is handled in the liver The individual steps of this process may be visualized as follows

- 1 Transport of bilirubin from the plasma into the liver cells
- 2 Localization of bilirubin at the surface of or penetration into liver cell microsomes
- 3 Conjugation of bilirubin by the microsomal enzyme system
- 4 Transport of conjugated bilirubin from the hepatic cells into the bile

A defect in one or more of the first three steps would result in decreased formation of conjugated bilirubin and hence in retention of *unconjugated* bilirubin in the plasma A disturbance of the fourth step would lead to impaired excretion of *conjugated* bilirubin resulting in its regurgitation into the plasma

In the following discussion a number of syndromes will be considered in which icterus appears to be due to one or a combination of several such defects It should be stated at the onset however that in most instances direct and unequivocal demonstration of the precise nature of the defect(s) has not yet been possible For the most part the argument is indirect or by analogy Moreover clear evidence for genetic control of the catabolic abnormalities is available only in some of the syndromes while in others inheritance may be suspected but has not yet been proved

## FORMATION AND METABOLISM OF BILIRUBIN

### BREAKDOWN OF HEMOGLOBIN

The average life span of the normal human erythrocyte is 120 days [19] At the end of this period the cell is removed from the circulation and its

## Chapter 8

### Hyperbilirubinemia\*

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Rudi Schmid

Jaundice as a manifestation of liver disease has been known for centuries. Its diagnostic and prognostic aspects were discussed by Hippocrates [1] and its association with the presence of gall stones in the biliary tract was recognized by Calen [2].

The concept that *bile* pigment is derived from *blood* pigment is probably very old, but the first clear and experimentally supported indication was provided by Virchow [3] who observed and isolated bilirubin crystals from old blood extravasations. Through many classical experiments conducted over the following years, unmistakable evidence accumulated that bilirubin is derived from the prosthetic group of hemoglobin [4-9]. The final proof was obtained much later when Fischer and his school elucidated the *chemical* constitution of heme and of bilirubin and demonstrated that both have a closely related tetrapyrrolic structure [10].

For a long time the anatomic site of bilirubin formation in the organism was a matter of much controversy. On the basis of the findings of Minkowski and Naunyn [11] that in hepatectomized guinea induced hemolysis failed to produce jaundice, it was thought that the liver was the only organ capable of converting hemoglobin to bilirubin. This was an unfortunate interpretation of a correct observation, later clarified by the finding that in the guinea virtually all reticuloendothelial cells are located in the liver. In mammals McNee [6], Mann [8], Rich [9] and others obtained conclusive evidence that formation of bilirubin does occur following total hepatectomy. As a result of these findings, the view gained general acceptance that hemoglobin is converted to bilirubin in the entire reticuloendothelial system, the pigment is then transported in the blood to the liver where it is excreted in the bile.

Quantitative studies of bile pigment metabolism were greatly facilitated when van den Bergh introduced Ehrlich's diazo reaction for estimation of

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Conversion of hematin to bile pigments has been demonstrated *in vivo* and *in vitro* [8 30-34]. The earlier belief that this occurs by formation of hematoporphyrin or protoporphyrin as intermediates has been shown to be erroneous [31]. Krench et al [33] failed to observe bile pigment formation *in vitro* by coupled oxidation of protoporphyrin and L-ascorbic acid whereas under the same conditions positive results were obtained with hematin, methemalbumin, hemoglobin, and methemoglobin. These findings and those reported by Fischer [35], Libowitzky [34], and Lemberg [36] suggest that the presence of a divalent or trivalent iron may be

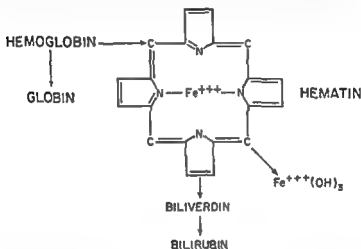


FIG 8-2 Hemoglobin catabolism. The exact nature of the breakdown of hemoglobin is not fully understood. According to one view, the first intermediate formed is hematin.

essential for the cleavage of the protoporphyrin ring. Recent studies *in vivo* [37] which originally were believed to demonstrate low grade conversion of isotopically labeled protoporphyrin to stercobilin do not contradict this concept since it is likely that a small fraction of the administered protoporphyrin is first converted to heme before being broken down to bile pigments [24]. It is well established that protoporphyrin may undergo spontaneous coordination with ferrous iron to form heme [38-39]. With the exception of a photochemical reaction requiring very special conditions [40] there is no evidence to suggest that free protoporphyrin can be directly converted to bile pigments *in vivo* or *in vitro*.

Lemberg and his school have challenged the concept that hematin is an intermediate in the physiologic breakdown of hemoglobin to bile pigments [36]. Coupled oxidation of hemoglobin and a corbic acid *in vitro* was found to result in formation of choleglobin, a green bile pigment-iron globin complex which upon treatment with acetic acid yields

hemoglobin is degraded [10]. The factors responsible for the "aging" of circulating red blood cells are poorly understood. Destruction of old erythrocytes takes place in the reticuloendothelial cells [19] but it is not known to what extent the different organs containing the reticuloendothelial cells participate in this process. Under conditions of abnormally increased erythrocyte destruction, cells may be destroyed predominantly either in the spleen or in the liver, depending upon the type of damage to which the erythrocytes are subjected [20]. The problems relative to the site of red cell destruction and bilirubin formation have been reviewed by Lefebvre [21]. With [11] and Watson [13].

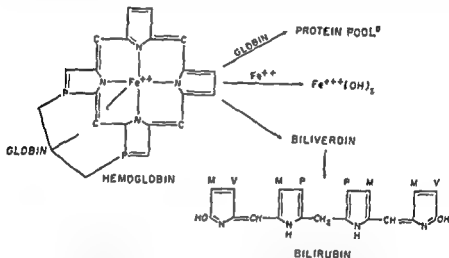


FIG 8-1 Hemoglobin catabolism. Of the three hemoglobin constituents the iron and the globin amino acids are reutilized whereas the protoporphyrin is converted to bile pigment and excreted.

Of the three hemoglobin constituents iron is almost completely reutilized for formation of new iron-containing compounds [22]. The globin is probably degraded and returned to the amino acid pool (Fig 8-1) [19]. The protoporphyrin moiety is not preserved; the porphyrin ring is opened and the resulting bile pigment is excreted (Fig 8-1) [23, 24]. The exact nature and the sequence of the individual steps in the catabolism of hemoglobin are still matters of controversy.

It has been assumed that the first intermediate formed is hematin. According to this view, the initial step consists in plating of the iron protoporphyrin complex from the globin (Fig 8-2) [2, 13, 25, 26]. In conditions associated with intravascular hemolysis [27] and in some instances of severe liver disease [13, 28] the plasma may contain hematin bound to albumin (methemalbumin) [29]. Under physiologic conditions hematin cannot be demonstrated in the blood, presumably because the breakdown of hemoglobin occurs inside the reticuloendothelial cells [2, 1]

there are other metabolic products of hemoglobin [24] A significant discrepancy has occasionally been noted between the estimated rate of hemoglobin breakdown and the amount of bile pigments recovered [14 50-52] This is illustrated by patients suffering from severe congenital nonhemolytic jaundice of the type described by Crigler and Najjar [43] (see below) Children with this disorder have a reduced capacity to form conjugated bilirubin and hence to excrete the pigment causing marked retention of bilirubin in the plasma [54 55] The bile although otherwise normal may be almost totally devoid of bile pigments and fecal urobilinogen excretion is very low [54] In spite of nearly complete inability to excrete bilirubin the degree of jaundice is not steadily increasing but remains essentially stationary [24] This would not be expected if bilirubin were the only metabolic end product of hemoglobin breakdown It is possible that heme or bilirubin or both may be degraded to and excreted as di- or monopyrrole but detection and isolation of these compounds is technically difficult

Dipyrrolic compounds belonging to the group of bilifucins, mesobilifucins and pentdyopent have been identified in and isolated from the feces of healthy individuals [56-58] Pentdyopent has occasionally been found in the urine of jaundiced patients [59 60] However the metabolic origin of these substance has not yet been established with certainty [58]

In addition dark pigments belonging to the mesobilifucin group are excreted in the urine of patients with a rare congenital syndrome in which increased hemolysis is associated with the presence of erythrocyte inclusion bodies [61] In these instances the urinary pigments are believed to be derived from the abnormal red cell but the nature of the metabolic defect in the erythrocytes is not yet known

With [62] and Lups and Meyer [63] suggested that dipyrroles of the bilifucin type occur in normal and in jaundiced serums but direct evidence to support this claim was not obtained Exposure of hemoglobin to hydrogen peroxide *in vitro* [64] or coupled oxidation with unsaturated fatty acids [65] results in formation of dipyrrolic fragments There is no convincing evidence that such mechanisms are of physiologic significance [2]

The scattered observations indicate that the exact mechanism of hemoglobin breakdown is still poorly understood but there can be little doubt that bilirubin and the pigments and chromogen that result from its further alteration in the intestine represent the major end products of heme catabolism [2 24 36]

#### FORMATION OF BILE PIGMENTS FROM SOURCES OTHER THAN MATURE ERYTHROCYTES

Attempts to correlate bilirubin excretion in the bile with the rate of erythrocyte destruction as determined by other methods suggested that 80 to 100 per cent of the degraded hemoglobin can be accounted for as

biliverdin and free iron (Fig 8-3) [36] According to their view the porphyrin ring is opened while the heme group is still attached to the protein moiety. Recently Mills [31] and Kuziro et al [32] confirmed and extended these observations and suggested that in circulating erythrocytes protective mechanisms may prevent excessive formation of choleglobin during life. Small amounts of choleglobin like pigments have been observed in normal rabbit erythrocytes [33] and in stored human red cells [34] as well as in red cells of rabbits treated with phenylhydrazine [35]. It has been suggested that the so-called "easily split iron" of erythrocytes may actually represent that portion of the red cell iron which is part of the choleglobin [36].

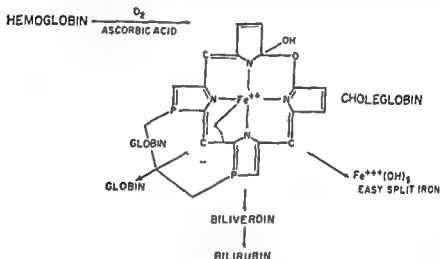


FIG 8-3 Hemoglobin catabolism. Another proposed mechanism for the breakdown of hemoglobin assumes that the first intermediate formed is choleglobin, which is a green bile pigment-iron globin complex in which the porphyrin ring has been opened in one of the methene bridges.

The demonstration of choleglobin like compounds in red cells is technically difficult because of the large excess of hemoglobin present. Whether the green pigments may simply represent an artifact is still a matter of considerable controversy [33, 37-40]. This question cannot be resolved at present. The finding of Kensch et al [33] that hematin can be converted in vitro to bile pigments makes it unnecessary to postulate that the opening of the porphyrin ring must take place while heme is still attached to protein. On the other hand, the fact that hematin may be converted to bile pigments is not proof that the former is a true intermediate in the breakdown of hemoglobin to bilirubin.

Whatever the physiologic pathway of hemoglobin catabolism may be, either with hematin or with choleglobin as intermediate, it appears certain that bilirubin is the principal bile pigment formed [2, 36]. There is at least suggestive evidence, however, that in addition to bilirubin

globin of mature circulating erythrocytes but must originate from other sources. It has been suggested that bile pigment may be formed from hemoglobin of developing erythrocytes which are destroyed in the marrow or shortly after they reach the circulation [67-68]. It is also possible that immature red cells may be *forming* as well as *degrading* hemoglobin [68] or that heme formed in excess of globin may be degraded to bile pigment [68]. Direct synthesis and excretion of bile pigments appears less likely but cannot be excluded with certainty.

A small fraction of excreted stercobilin is undoubtedly derived from heme compounds other than hemoglobin such as myoglobin, catalase, peroxidase and cytochromes. The quantity of these chromoproteins in the organism is relatively small and little is known of their rate of turnover [69]. It has been suggested that nonhemoglobin heme may be the source of the small but significant amount of labeled stercobilin which is excreted between the thirtieth and eightieth day [67-68] following the administration of glycine- $N^{14}$  (Fig. 8-4). Since during this period there is no apparent decrease in the isotope concentration of circulating red cell hemoglobin, it appears unlikely that hemoglobin is the source of this bile pigment fraction.

Quantitative analysis of the pattern of labeled stercobilin excretion in normal individuals suggests that approximately 70 per cent of the excreted bile pigment originates from destruction of mature red cells which have reached the end of their physiologic life span [67-68]. An additional 10 to 20 per cent of pigment is excreted during the first week and comprises the initial peak [67-68]. This fraction, although probably not derived from hemoglobin of mature circulating erythrocytes, appears in some way to be related to formation or maturation of erythroid cells in the bone marrow [70]. The excretion of labeled stercobilin during the period between the thirtieth and the eightieth day amounts to less than 10 per cent of all bile pigments formed and probably reflects to a large extent turnover of heme compounds of nonhemoglobin nature.

In diseases associated with abnormalities in erythropoiesis, the pattern of bile pigment excretion may differ significantly from that found in healthy individuals. Thus in congenital erythropoietic porphyria at least 31 per cent [71] and perhaps as much as 80 per cent [68] of the excreted stercobilin is not derived from hemoglobin of mature circulating erythrocytes. Likewise in pernicious anemia 40 per cent or more of the fecal bile pigment seems to be derived from sources other than mature red blood cells [72].

These findings indicate that the excreted bile pigments are derived from multiple metabolic sources, the most important of which is undoubtedly hemoglobin in circulating erythrocytes. On the other hand, estimation of pigment excretion in bile or feces may fall short of the total turnover of heme compounds in the body since alternative pathways



bile pigments [66]. This concept, based on studies carried out *before* the advent of tracer techniques requires modification now that it is known that a significant fraction of the excreted bile pigment is derived from sources other than hemoglobin of aged erythrocytes.

When glycine- $N^{15}$  is administered to a normal person and the concentration of the labeled nitrogen is determined in the excreted bile

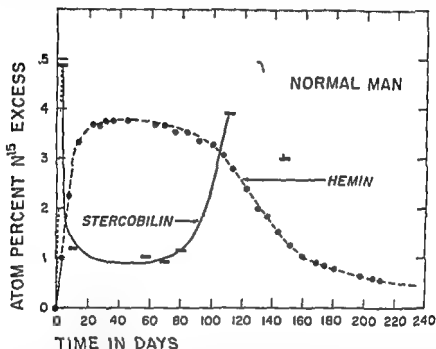


FIG 8-4 Formation of bile pigment in normal man. When glycine  $N^{15}$  is administered to normal man, hemin of circulating hemoglobin shows a rapid increase in isotope content. Fecal stercobilin exhibits two separate peaks of high isotope concentration: a first maximum is reached during the first week, while the second maximum coincides with the period of maximal breakdown of labeled erythrocytes. (By permission of I. M. London et al. [67].)

pigments over the following 150 days a curve with two separate peaks is obtained, an initial smaller peak is reached during the first week after the isotope administration and a second larger peak occurs after approximately 130 days [67, 68] (Fig 8-4). The second peak coincides with the period of maximal breakdown of labeled erythrocytes (Fig 8-4), suggesting that this fraction of fecal stercobilin is derived from mature red cells which are destroyed at the end of their physiologic life span.

The initial peak in  $N^{15}$  labeled stercobilin occurs at a time when the  $N^{15}$  of hemoglobin in circulating red cells is still increasing (Fig 8-4). This fraction of stercobilin could not therefore be derived from hemo-

curonic acid is attached to the carboxyl groups of the pigment to form an acyl glucuronide (Fig 8 6) which is unstable at alkaline pH [83 84] Because of its water solubility conjugated bilirubin exhibits a *direct* van den Bergh reaction while unconjugated bilirubin couples with the diazo reagent only after addition of alcohol or of another suitable solvent (*indirect* van den Bergh reaction) [81 82] It should be noted, however that in plasma a small fraction of the unconjugated pigment may react with the diazo reagent in the *absence* of alcohol [14 82] This is probably because of the presence of solubilizing substances such as bile acids urea

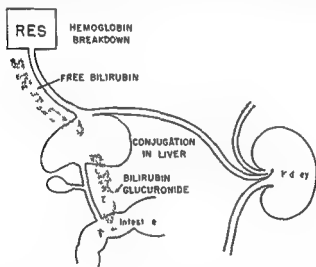


Fig 8 6 In man and other mammals bilirubin is excreted in conjugated form mainly as a glucuronide

and citrate [14] For this reason the direct and indirect van den Bergh reactions do not permit an exact estimation but only an *approximation* of the concentration of conjugated and unconjugated bilirubin present [82]

Many metabolites and several exogenous compounds administered for diagnostic or therapeutic reasons are excreted as glucuronide. Glucuronide formation provides the organism with a mechanism by which harmful or metabolically active substances can be detoxified or inactivated. Synthesis of glucuronides is catalyzed by an enzyme system present in liver microsomes [85-87] It is not yet known whether a single enzyme or several related microsomal enzymes are involved in the biosynthesis of different types of glucuronides [88] In all instances the source of the glucuronic acid moiety is the nucleotide uridine diphosphate glucuronic acid (UDPGA) formed by a dehydration reaction catalyzed by an

of catabolism may exist and may yield metabolic products not detected with the available methods [24]

#### TRANSPORT, CONJUGATION, AND EXCRETION OF BILIRUBIN

The first bile pigment formed in the reticuloendothelial cells is probably biliverdin [2-36] which is rapidly reduced to bilirubin. The bile of many species of Amphibia and birds contains only biliverdin [2] but mammals excrete predominantly bilirubin [36]. Reduction of biliverdin to bilirubin *in vitro* has been demonstrated in a variety of mammalian tissues including liver, kidney and spleen [36, 37].

The exact mechanism by which bilirubin is released from the reticuloendothelial system into the plasma is not clear. Since the pigment is *insoluble* at the pH of the blood [18] it must be carried by attachment to proteins. The vast and contradictory literature relating to the mode of transport of bilirubin in the plasma has been reviewed by With [14]. It is generally agreed that in jaundiced serums most of the bilirubin is bound to albumin [14] but attachment of bilirubin to other plasma proteins has also been demonstrated [74]. Cohn [75] has isolated a pure  $\alpha_1$  globulin belonging to the V<sub>1</sub> fraction which strongly binds bilirubin. This fraction was estimated to constitute less than 0.1 per cent of all plasma proteins [76].

It should be noted in this regard that most investigations on the mode of protein binding of bilirubin were carried out either with icteric serum or with serum to which bilirubin had been added; the finding that under the *in vivo* conditions albumin appeared to be the principal carrier does not necessarily indicate that the same protein fraction is responsible for the transport of the much smaller amounts of bilirubin which are present under *physiologic* conditions. Indeed Cohn's observation [75] may suggest that the  $\alpha_1$  globulin fraction perhaps contains a specific bilirubin binding protein.

In healthy individuals the serum bilirubin concentration ranges from 0.1 to approximately 1.5 mg per 100 ml [76] but most values lie between 0.3 and 1.0 mg per 100 ml [14]. Many animals such as rats, mice, dogs and cats have much lower values [14] while in horses the concentration is higher than in man.

For excretion in the bile bilirubin is converted to water soluble conjugates [15-17] (Fig. 8.5). In man [15-17], cat [77], rat [78], guinea pig [79], dog [17] and sheep [80], most of the bilirubin in the bile is present as diglucuronide while a smaller fraction is excreted as monoglucuronide [81]. In addition a minor pigment fraction is present as sulfide [77]. Human bile contains trace amounts of other water soluble forms of bilirubin the nature of which is not yet known [77].

Glucuronide formation is the major pathway by which bilirubin is rendered water soluble and suitable for excretion [77-81, 82]. The glu-

Bio synthesis of bilirubin glucuronide *in vitro* has been demonstrated with slices [90] homogenates [90 91] and microsomal preparations [84] of rat liver. Rat liver preparations also catalyze formation of bilirubin sulfate [77]. In addition to liver, other tissues have the ability to form glucuronides *in vitro*, particularly the kidney [92] and the mucosa of the upper gastrointestinal tract [92]. Nevertheless, *in vivo* extrahepatic conjugation of bilirubin is probably of little physiologic significance, although Bollman has suggested that in hepatectomized animals, bilirubin monoglucuronide may be formed in organs other than the liver [93].

### THE PATHOPHYSIOLOGY OF HYPERBILIRUBINEMIA

In a general way, hyperbilirubinemia occurs as the result of two unrelated but frequently associated disturbances, namely, retention in

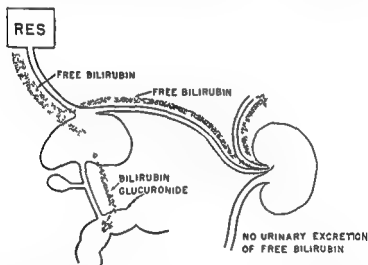


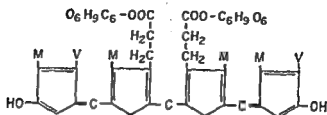
Fig 8-8 In hemolytic jaundice the pigment load exceeds the capacity of the liver to handle bilirubin.

the plasma of *unconjugated* pigment and regurgitation into the plasma of bilirubin which has already been *conjugated*. The former group includes the hemolytic syndromes and the instances of jaundice where metabolic defects prevent adequate conjugation of bilirubin. The second group comprises the forms of icterus associated with *extrahepatic obstruction* of the bile ducts and with *intrahepatic (functional) cholestasis*.

In hemolytic jaundice, the accelerated rate of red cell destruction leads to overproduction of bilirubin in amounts which may exceed the capacity of the liver to handle the pigment (Fig 8-8) [81 82]. It has been shown, however, that under experimental conditions the ability of normal man [14] or rat liver [9,] to conjugate and excrete injected bili-

## CONJUGATED BILIRUBIN

## DIRECT REACTING\*



## FREE BILIRUBIN

## "INDIRECT - REACTING"

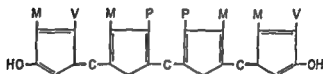


Fig 8-6 In conjugated bilirubin glucuronic acid is attached to the propionic acid radicals of bilirubin to form an  $\alpha$ -glucuronide

## ENZYMATIC FORMATION OF BILIRUBIN DIGLUCURONIDE

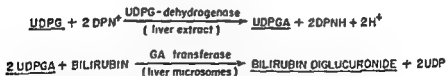


Fig 8-7 Glucuronic acid is transferred from the nucleotide uridine diphosphate glucuronic acid (UDPGA) to bilirubin. UDPGA is formed by dehydrogenation of uridine diphosphate glucose (UDPG)

soluble enzyme system which is present in liver [85-87] (Fig 8-7). Glucuronic acid in its free form cannot be incorporated into glucuronides but there is suggestive evidence at least in vitro that UDPGA may be formed from uridine triphosphate and glucuronic acid 1-phosphate in the presence of ATP [89].

## NEONATAL HYPERBILIRUBINEMIA

In the liver of fetal mice [97] rabbits [98] rats [99] and guinea pigs [79-92] the rate of formation of glucuronides is much reduced as compared with that of adult animals. The same is true of the fetal kidney [92] but apparently not of the gastrointestinal mucosa [92]. This reduction appears to involve both synthesis of uridine diphosphate glucuronic acid and activity of the glucuronyl transferase system in the microsomes [79-92]. Towards the end of the gestational period fetal tissues exhibit a slow increase in the rate of glucuronide formation but at birth the enzymatic activity still is severalfold below that of adult animals (Fig 8-9).

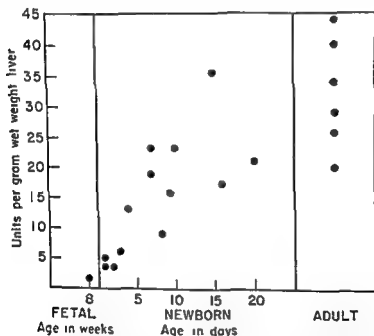


Fig 8-9 Glucuronide formation in fetal neonatal and adult liver. Formation of *o*-aminophenyl glucuronide was estimated in liver homogenate of fetal neonatal and adult guinea pigs. Similar results were obtained with liver homogenate of other mammals (Reproduction of L. A. Boyer et al [10]).

During the immediate neonatal period the magnitude of glucuronide formation increases rapidly; it reaches adult levels a few days to 2 weeks after birth [79-92-98]. Attempts to induce increased enzymatic activity in fetal liver by applying the pregnant rat with large amounts of an aglycone substrate were unsuccessful even though the substrate passed the placental barrier and accumulated in the fetal liver [100] (Table 8-1). Similar observations have been made with other microsomal enzyme

rubin is so great that in all but the most severe instances of hemolysis it is difficult to understand how increased breakdown of hemoglobin could be the sole cause for the observed retention of pigment in the plasma. This discrepancy has been explained by assuming the coexistence of a relatively specific derangement in liver function. It is also possible that in instances of chronic hemolysis the removal of bilirubin from the plasma may be limited by the rate at which the pigment can be delivered to the liver cells; such a limitation could result from alterations in the plasma protein fractions responsible for the transport of bilirubin.

The icteric syndromes associated with retention of unconjugated bilirubin in the absence of significant hemolysis are discussed below.

In acute extrahepatic obstruction of the bile ducts the predominant pigment in the plasma is bilirubin diglucuronide [93, 94] but smaller increases in monoglucuronide and in unconjugated bilirubin are often also present [93, 94]. In chronic obstruction bilirubin monoglucuronide is frequently found in larger amounts than diglucuronide or unconjugated bilirubin [93, 94]. This is believed to be due to variable degrees of hepatic cell dysfunction in addition to the obstruction [81].

In hepatitis and in cirrhosis the plasma usually contains both conjugated and unconjugated bilirubin [41, 82]. Thus in addition to the impairment in hepatocellular function the same conditions are associated with a defect in excretory function, but this explanation is not entirely satisfactory. Future studies on the fine structure of the liver and particularly on the mechanisms involved in the excretion of the various bile constituents may reveal information requiring considerable modification of present concepts.

## HYPERBILIRUBINEMIA ASSOCIATED WITH DEFECTIVE FORMATION OF CONJUGATED BILIRUBIN

In human bile and in that of other mammals essentially all bilirubin is present in conjugated form, mostly as mono- or diglucuronide. Conjugation is probably essential for the excretion of bilirubin in the bile [78]. Thus it would be expected that impaired formation of conjugated bilirubin results in reduced pigment excretion and hence in retention of unconjugated bilirubin in the plasma. As mentioned earlier, such a defect may involve the transport of bilirubin into the liver cells, the penetration of the pigment into the subcellular microsomes, or the activity of the microsomal enzyme system catalyzing the actual conjugation.

A transient type of hyperbilirubinemia, probably due to reduced activity of the glucuronide-forming enzyme system, is frequently observed during the first days of the neonatal period [96]. Hereditary forms of icterus associated with similar enzymatic defects are found in man [54, 55] and in the rat [78].

this pathway and results in transient hyperbilirubinemia which persists until the neonatal liver reaches the functional capacity to handle its own pigment load

#### HEREDITARY HYPERBILIRUBINEMIA IN RATS (GUNN STRAIN)

Gunn [102] and Malloy and Loewenstem [103] described a mutant strain of Wistar rats exhibiting hereditary acholuric jaundice. The disturbance is probably inherited as a Mendelian recessive trait since litters produced by nonicteric parents known to be carriers of the trait



Fig 8-10 Biliary excretion of Cholografin in rat with hereditary hyperbilirubinemia. The contrast medium was demonstrable by x ray in the small bowel 90 min after its intravenous injection. Rats lack a gallbladder.

usually include 25 per cent icteric offspring [102]. Icteric animals exhibit bilirubin levels ranging from approximately 5 mg per 100 ml to well above 20 mg. All plasma bilirubin is of the unconjugated type and bilirubinuria is absent [78]. There is no evidence of increased hemolysis [103] and liver morphology is normal [78]. In rats with bilirubin level exceeding 12 to 15 mg per 100 ml, functional impairment and morphologic alterations of the central nervous system resembling kernicterus are frequently observed [104].

The biliary excretory system is patent as demonstrated by rapid excretion of radiopaque contrast medium (Cholografin) and of Bromsulphalein [78] (Fig 8-10). Moreover, conjugated bilirubin injected intravenously is rapidly and completely excreted in the bile with biliary pigment concentration 100 times one hundred fold (Fig 8-11) [78]. Maximal clearance for conjugated bilirubin in jaundiced rats is similar to the maximal clearance for unconjugated bilirubin in normal rats as determined



systems which are "underdeveloped" in the fetus and reach their full activity only after birth [101]

In man estimates of the magnitude of glucuronide formation in fetal and newborn liver are difficult to obtain. Dutton [92] studied the liver of two human fetuses aged 3 to 4 months and found very low activity of both glucuronyl transferase and uridine diphosphate glucose dehydrogenase. In the liver of three prematurely born infants (birth weight from 766 to 1312 gm), Lathe and Walker observed only negligible glucuronide formation [90]. These two reports together with the more extensive observations in other mammalian species, suggest that the inadequate development of the glucuronide-forming enzyme system at

TABLE 8-1 BIOSYNTHESIS OF *o*-AMINOPHENYL GLUCONIDURONIC ACID BY MICROSOMES OF MATERNAL AND FETAL RAT LIVER AFTER REPEATED INJECTION OF *o*-AMINOBENZOATE TO THE MOTHER

| Type of treatment       | No of animals | <i>o</i> -Aminophenyl glucuroniduronic acid formed $\mu$ moles/gm |                   |
|-------------------------|---------------|---|-------------------|
|                         |               | Mother  | Fetus             |
| <i>o</i> -Aminobenzoate | 6             | 0.011 $\pm$ 0.013   | 0.009 $\pm$ 0.003 |
| Saline controls         | 6             | 0.011 $\pm$ 0.019   | 0.011 $\pm$ 0.003 |

SOURCE: R. Schmid et al. [100]

birth is probably the most important factor in producing the transient hyperbilirubinemia in the newborn. In infants with erythroblastosis fetalis the increased pigment load resulting from the hemolytic process is superimposed upon this functional defect in the liver.

The fate of the bilirubin produced by the fetal organism during the prenatal period has been a matter of great interest. Since the fetal conjugating apparatus is operating at a low level of efficiency, significant excretion of bile pigment into the intestinal tract cannot occur. On the other hand, the bilirubin level of fetal blood is usually low, but it rises steeply after severance of the umbilical cord [96]. These observations point to the placenta as the organ responsible for clearing the fetal blood of excess bilirubin. Until recently it was not known whether the fetal pigment is conjugated in the placenta or whether it is transferred to the maternal circulation for conjugation in the maternal liver. In human and rat placenta no evidence for the presence of a functioning glucuronide-forming mechanism was obtained [100]. On the other hand, it was found that in guinea pigs unconjugated bilirubin can be rapidly transferred from fetal blood through the placenta to the maternal circulation [100]. These observations suggest that bilirubin produced in the fetus may reach the maternal blood unchanged and may then be conjugated and excreted by the maternal liver. Severance of the umbilical cord interrupts

this pathway and results in transient hyperbilirubinemia which persists until the neonatal liver reaches the functional capacity to handle its own pigment load

#### HEREDITARY HYPERBILIRUBINEMIA IN RATS (GUNN STRAIN)

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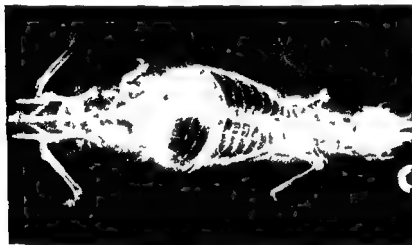


Fig 8-10 Biliary excretion of Cholografin in rat with hereditary hyperbilirubinemia. The contrast medium was demonstrable by x ray in the small bowel 20 min after its intravenous injection. Rats lack a gallbladder.

usually include 25 per cent icteric offspring [102]. Icteric animals exhibit bilirubin levels ranging from approximately 5 mg per 100 ml to well above 20 mg. All plasma bilirubin is of the unconjugated type and bilirubinuria is absent [78]. There is no evidence of increased hemolysis [103] and liver morphology is normal [78]. In rats with bilirubin levels exceeding 12 to 15 mg per 100 ml functional impairment and morphologic alterations of the central nervous system resembling kernicterus are frequently observed [104].

The biliary excretory system is patent as demonstrated by rapid excretion of radiopaque contrast medium (Cholografin) and of Bromsulphalein [78] (Fig 8-10). Moreover conjugated bilirubin injected intravenously is rapidly and completely excreted in the bile with biliary pigment concentration rising one hundred fold (Fig 8-11) [78]. Maximal clearance for conjugated bilirubin in jaundiced rats is similar to the maximal clearance for unconjugated bilirubin in normal rats as determined

by Weinreb and Billing [9]. In icteric rats on the other hand in intravenous administration of unconjugated bilirubin results in biliary excretion of less than 1 per cent of the amount excreted by normal rats with a comparable serum bilirubin level [7].

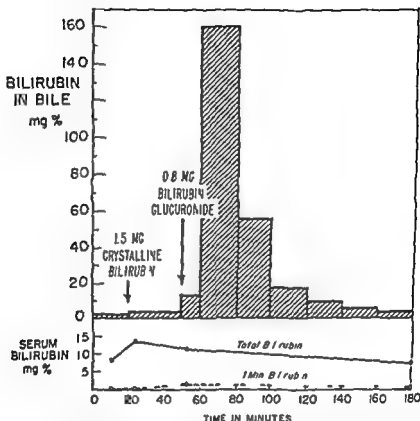


Fig 8-11 Biliary excretion of injected bilirubin in rat with hereditary hyperbilirubinemia. Crystalline bilirubin and conjugated bilirubin were injected in a tail vein of a jaundiced rat. The conjugated pigment was rapidly excreted in the bile while injection of crystalline bilirubin resulted only in a highly increased concentration of nonconjugated bilirubin. (By permission of R. Schindler et al. [8].)

The bile of icteric rats contains much less bilirubin than that of animals without jaundice and the small amount of pigment present is in the nonglucuronide form [78]. Furthermore fecal urobilinogen excretion in jaundiced rats is greatly reduced as compared with that in control animals [78] (Table 8-2) but the color of the feces is normal.

Total urinary excretion of glucuronic acid and glucuronides is approximately half of that in control animals [78]. When compounds that are normally excreted as glucuronides are administered to icteric rats the rise in urinary glucuronide excretion is significantly smaller than in con-

TABLE 8-9 FECAL UROBILINOGEN EXCRETION IN WISTAR RATS

| Rats                                    | No of animals | Urobilinogen<br>mg/day/100 gm body weight |                    |
|---|---------------|---|--------------------|
|   |               | Mean                                      | Standard deviation |
| Jaundiced (Gunn strain)                 | 3             | 0.003                                     | $\pm 0.004$        |
| Nonjaundiced litter mates (Gunn strain) | 3             | 0.039                                     | $\pm 0.013$        |
| Normal control                          | 4             | 0.077                                     | $\pm 0.04$         |

SOURCE: R. Schmid et al [78]

control animals [78] (Fig 8-12). This is observed both with aglycones that form etheral glucuronides such as menthol and with ester-forming aglycones such as  $\alpha$ -aminobenzoic acid [78]. Likewise injection of benzoate to jaundiced rats results in urinary excretion of larger amounts of hippurate and smaller amounts of benzoyl glucuronide as compared with excretion of these substances in nonicteric animals [78].

#### ANTHRANILIC ACID GLUCURONIDE EXCRETION AFTER ANTHRANILIC ACID ADMINISTRATION (25 MG PER 100 GM RAT)

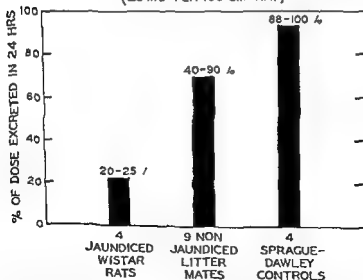


Fig 8-1. Urinary excretion of  $\alpha$ -aminobenzoic acid glucuronide was estimated after intraperitoneal injection of a loading dose of  $\alpha$ -aminobenzoate (anthranilate). In jaundiced rats a much smaller fraction of the administered compound was excreted in the form of the glucuronide as compared with normal rats. Nonjaundiced littermates of the same animal excreted less glucuronide than genetically normal rats but more than control rats. (By permission of R. Schmid et al [8].)

Glucuronide formation with liver of jaundiced rats was studied *in vitro* by incubating slices [90] homogenates [90 100] and microsomal preparations [50, 78]. In all instances, glucuronide synthesis was reduced but the results differed depending on the substrate used for the enzymatic assay. With bilirubin as substrate formation of bilirubin glucuronide could not be detected [100] whereas with *o*-aminophenol *o*-aminophenyl glucuronide was formed but at a greatly reduced rate [78 90] (Table 8-3).

TABLE 8-3 ENZYMIC SYNTHESIS OF URIDINE DIPHOSPHATE GLUCURONIC ACID (UDPGA) AND OF *o*-AMINOPHENYL GLUCURONIC ACID BY MALE RAT LIVER MICROSOMES

| Rats                      | No. of animals | UDPG | <i>o</i> -Aminophenyl glucuronic acid |
|---------------------------|----------------|------|---------------------------------------|
| Jaundiced                 | 6              | 4.9  | 0.008                                 |
| Nonjaundiced litter mates | 8              | 4.3  | 0.031                                 |
| Normal controls           | 7              | 5.8  | 0.061                                 |

Results expressed in micromoles formed per gram of liver

SOURCE: R. Schmid et al. [78]

In jaundiced rats the activity of uridine diphosphate glucose dehydrogenase in the liver was comparable to that in normal animals [78]. Addition of excess uridine diphosphate glucuronic acid to microsomal preparations of icteric rat liver failed to augment glucuronide synthesis [78 90]. In kidneys of icteric rats, a comparable reduction in glucuronide formation was observed [78 105].

These findings suggest that the primary defect in jaundiced rats involves the glucuronyl transferase system in the microsomes. It remains unexplained, however, why glucuronide formation both *in vivo* and *in vitro* is not demonstrable with bilirubin whereas with other compounds small but significant amounts of the glucuronides are formed [78 90]. It has been suggested that bilirubin may be unsuccessful in competing with other aglycones for the deficient glucuronide-forming enzyme system [55] but this explanation is difficult to reconcile with the observation that injection of *o*-aminobenzoate does result in increased glucuronide excretion (though the increase is smaller than in nonicteric animals (Fig. 8-12)) whereas bilirubin has no such stimulatory effect. Another possible explanation is that the formation of different types of glucuronides is catalyzed by separate microsomal enzyme systems and that in jaundiced rats only the enzyme involved in the synthesis of bilirubin glucuronide is deficient. However, this is not borne out by the experimental findings in that formation of glucuronides is reduced with all aglyconic compounds that have been tested.

An attempt was made to stimulate the activity of the glucuronide-forming enzyme system in jaundiced rats by repeated administration of

large amounts of an aglyconic substrate other than bilirubin [80] For 5 consecutive days 50 mg per 100 gm body weight of sodium *o*-aminobenzoate was injected twice a day by intraperitoneal route At the end of this period liver microsomes prepared from these animals failed to exhibit increased activity of glucuronyl transferase as compared with similar preparations from icteric rats injected with equivalent amounts of sodium chloride In the icteric rats treated with *o*-aminobenzoate the liver exhibited considerable concentrations of the administered compound as long as 20 hr after the last injection; whereas in nonicteric control animals the injected *o*-aminobenzoate was conjugated and removed from the liver within a few hours [80] These findings indicate that the defect in glucuronyl transferase activity cannot be corrected by supplying excessive amounts of substrate This conclusion could be anticipated because the increased concentration of circulating bilirubin would be expected to provide an already adequate stimulation for enzyme induction

From the above discussion it is apparent that the hyperbilirubinemia in this strain of rats results from an enzymatic defect which precludes conversion of bilirubin to its water soluble glucuronide and hence interferes with the excretion of the pigment in the bile A defect of this type had already been anticipated by Malloy and Loewenstein [103]

It is of interest that in spite of this severe impairment in pigment excretion the physiologic turnover of heme compounds does not result in a steadily increasing degree of jaundice It must be concluded that alternate pathways for heme or bilirubin catabolism must exist [78] which at these increased bilirubin levels are capable of establishing a new balance between formation and breakdown of pigment

In nonicteric rats which are known carriers of the jaundice trait glucuronide formation *in vivo* and *in vitro* is reduced as compared with that in genetically normal rats but is greater than that in icteric rats [78-80] Thus in animals which are heterozygous for the trait the metabolic abnormality is present in a less severe degree and is insufficient to give rise to pigment retention in the plasma

This strain of icteric rats provides an unusual opportunity for the study of bilirubin metabolism and they are an excellent experimental tool for investigations into the pathogenesis of kernicterus [104]

#### CONGENITAL FAMILIAL NONHEMOLYTIC JAUNDICE IN MAN (CRIGLER NAJJAR SYNDROME)

##### *Clinical Findings*

A familial form of severe nonhemolytic jaundice associated with disturbances of the central nervous system was described in 1952 by Crigler and Najjar [63] In three related families a total of 14 infants was ob-

served in whom severe icterus appeared on the first or second day after birth and persisted throughout lifetime. There was no evidence of increased hemolysis or of blood group incompatibilities. The serum bilirubin was virtually all of the indirect reacting type and bilirubinuria was absent. Liver histology and conventional liver function tests failed to reveal significant abnormalities.

All but one of the 11 icteric infants developed severe disturbances of the central nervous system resembling kernicterus, and all five died during the first 15 months of life. In one instance where the brain could be examined at autopsy the cerebral cortex and the basal ganglia showed intense staining with bile pigment. The sixth icteric child of this family group failed to develop neurologic abnormalities [106] and at the time of this writing is 8 years old and well except for persistent jaundice. It is not known why only one of the six affected children escaped neurologic damage but it may be significant that during the first months of life this child's serum bilirubin level was somewhat lower than those of the other infants in whom serial determinations were reported [53].

An additional case belonging to the same family group was discovered in 1956 [106]. This now 5 year old girl, a double first cousin of the other patients, developed icterus on the first or second day of life. Serum bilirubin levels of approximately 25 mg per 100 ml have persisted since birth but neurologic disturbances have so far not been detected [106].

A few other cases exhibiting similar features have been observed and in part reported in the literature. Rothenthal et al. [107] studied a 5 year old boy who became icteric on the second day of life. The serum bilirubin concentration has remained between 20 to 35 mg per 100 ml and almost all of it gives an indirect van den Bergh reaction. Bilirubinuria was never present. Despite the persistent icterus the child remained well until the age of 3 when slight slurring of speech and intention tremor were noticed. At the age of 5 years in the course of a few weeks severe disturbances of motor function developed with ataxia and severe dysarthria. When last seen at the age of 9 the boy was severely crippled.

Both parents and a younger brother of this patient have normal bilirubin levels [107]. A striking feature of the family history is that the mother's brother and the father's sister, who are married to each other, had a first child who died at the age of 1 week with jaundice and central nervous system disturbances. At necropsy no evidence for hemolytic or hepatobiliary disease could be detected; unfortunately, the brain was not examined.

The only patient with this syndrome believed to have reached adult life was reported by Jervis [108]. This woman, who lived the last 30 years of her life in a mental institution, died from pneumonia at the age of 44. At the age of 11 months the patient was diagnosed as having 'Little's disease with idiocy and jaundice'. On admission to the mental institution

he exhibited deep jaundice, severe muscular rigidity, dystonia, partial deafness, and mental deficiency. The manifestations did not progress but the bilirubin level remained between 16 to 22 mg per 100 ml. At autopsy the liver was found to be grossly and histologically normal but the brain was reduced in weight and on histologic examination exhibited degenerative changes consistent with kernicterus.

Klingberg et al [109] observed two siblings with persistent severe icterus and central nervous system manifestations, both of whom died during the first year of life. At autopsy, no evidence for increased hemolysis, liver disease, or biliary obstruction could be detected.

TABLE 8-4 CLINICAL AND LABORATORY FINDINGS IN PATIENTS WITH CONGENITAL NONHEMOLYTIC JAUNDICE

| Patient and reference | Sex | Age icterus noted | Total serum bilirubin (range mg/100 ml) | Findings of neurologic in children | Condition in 1953 |
|-----------------------|-----|-------------------|---|------------------------------------|-------------------|
| J M [9]               | F   | 2 days            | 29-37                                   | Clinical                           | Died at 21 wk     |
| J R H [3]             | M   | 2 days            | 26                                      | Clinical and anatomic              | Died at 18 days   |
| J D H [106]           | M   | 3 days            | 17-27                                   | None                               | Living at 8 yr    |
| J I T [3]             | M   | 3 days            | 11-33                                   | Clinical                           | Died at 16 mo     |
| L T [53]              | M   | 3 days            | 13-40                                   | Clinical                           | Died at 23 wk     |
| J J T [53]            | M   | Birth             | Icterus in lex 20                       | Clinical                           | Died at 47 wk     |
| M L H [106]           | F   | 1 day             | 25                                      | None                               | Living at 5 yr    |
| J D [10]              | M   | 2 days            | 20-35                                   | Clinical                           | Living at 9 yr    |
| [109]                 | F   | 6 mo              | 16-22                                   | Clinical and anatomic              | Died at 44 yr     |
| J d E [90]            | M   | 3 days            | 20-25                                   | None                               | Living at 2½ yr   |
| R S [90]              | F   | 1 day             | 16-20                                   | None                               | Living at 8 yr    |
| W J H [104]           | M   | 3 days            | 15-35                                   | Clinical and anatomic              | Died at 1 yr      |
| J H [109]             | F   | 3 days            | 20                                      | Clinical and anatomic              | Died at 1 yr      |

Early observation not reported [105]

In addition to the case the author is following two patients with severe persistent jaundice. Both are similar to those described above except that to date they have escaped detectable damage to the central nervous system. The older (R S) a girl of 8 with a bilirubin concentration between 16 to 20 mg per 100 ml underwent two exploratory laparotomies which revealed normal liver histology and patency of the biliary tree. The younger is a boy of two (J d E) whose bilirubin concentration ranges from 20 to 25 mg per 100 ml. These cases are summarized in Table 8-4.

The author is indebted to Drs I K Hannon and L S Celli for permission to study these patients.



*Laboratory Examinations*

In none of the six patients was there evidence of hemolytic anemia as shown by normal hemoglobin concentration, low reticulocyte counts, normal bone marrow morphology, and absence of splenomegaly. The liver was not enlarged and on histologic examination, the only abnormality found was the presence of bile thrombi in hepatic canaliculi in



Fig. 8-13 Visualization of the gallbladder in patients with congenital nonhemolytic jaundice. In a patient with congenital nonhemolytic jaundice exhibiting a serum bilirubin concentration of 35 mg per 100 ml, intravenous cholangiography showed normal visualization of the gallbladder. (Courtesy of Dr. I. M. Leenthal, Chicago.)

some of the cases [53]. Conventional liver function tests including clearance of Bromsulphalein yielded uniformly normal results. In one child a low value for urinary excretion of hippuric acid was observed after oral administration of sodium benzoate [107], when this test was later repeated with intravenous sodium benzoate, hippuric acid excretion in the urine was within normal limits [80].

In two cases intravenous cholangiograms [80, 107] showed normal visualization of the gallbladder and the biliary ducts (Fig. 8-13). In two patients patency of the extrahepatic bile ducts was established at laparotomy.

The urine contained no bilirubin as shown by negative Gmelin Fouchet, and van den Bergh tests. In the four cases studied by the author the urine was of a golden yellow color which significantly differed in shade and intensity from that of normal urine. The nature of these yellow pigments is not known but they exhibited some similarities to those present in the plasma and urine of hepatectomized dog [110].

Bile was obtained from the duodenum of four children before and after stimulation of bile flow with Decholin or magnesium sulfate [54-80].

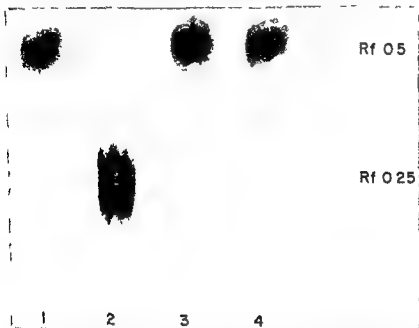


Fig 8-14 Bilirubin in serum of patients with congenital nonhemolytic jaundice. Ascending paper chromatogram of the azoderivatives of serum bilirubin from three patients with congenital nonhemolytic jaundice. All three serums contain only nonconjugated bilirubin ( $R_f$  0.5). The azoderivative of bilirubin diglucuronide has an  $R_f$  of 0.25. (1) Case J D male. (2) Control bilirubin diglucuronide. (3) Case J H female. (4) Case J D II male. (By permission of P. Schmidt [54].)

In three the bile specimens had a faint lemon juice color and bilirubin concentrations ranged from 0.1 to 1.0 mg per 100 ml. Essentially all of the pigment was present in unconjugated form. In all three fecal urobilinogen excretion was greatly reduced, ranging from 0.23 mg per 24 hr (2 years old) to 0.5 mg per 24 hr (7 years old) while serum bilirubin concentrations were in excess of 20 mg per 100 ml.

In the fourth child ( $R_S$ ) with a serum bilirubin of 17 mg per 100 ml the duodenal bile contained 4 mg bilirubin per 100 ml and about half was in the form of the glucuronide. This was later confirmed on lap-

*Laboratory Examinations*

In none of these patients was there evidence of hemolytic anemia as shown by normal hemoglobin concentration, low reticulocyte counts, normal bone marrow morphology, and absence of splenomegaly. The liver was not enlarged and on histologic examination, the only abnormality found was the presence of bile thrombi in hepatic canaliculi in



Fig. 8-13 Visualization of the gallbladder in patients with congenital nonhemolytic jaundice. In a patient with congenital nonhemolytic jaundice exhibiting a serum bilirubin concentration of 35 mg per 100 ml, intravenous cholangiography showed normal visualization of the gallbladder. (Courtesy of Dr. J. W. Rosenthal, Chicago.)

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# PLASMA LEVELS OF N-ACETYL-p-AMINOPHENOL AND OF N-ACETYL-p-AMINOPHENOL GLUCURONIDE

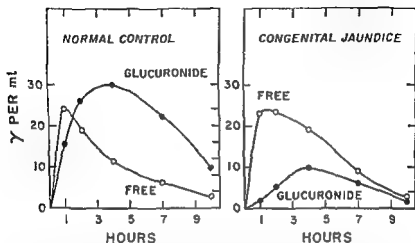


Fig 8-15 A male child with congenital nonhemolytic jaundice and a control subject of the same age and sex were given an oral dose of 30 mg per kg N acetyl p-amino phenol (NAPA). The concentration of NAPA glucuronide in the plasma of the icteric patient was much smaller than in the normal control (By permission of J Izetod et al [55])

a partial deficiency in the conjugation of reduced steroids with glucuronic acid

Similar results were obtained after oral administration of menthol [54] chloral hydrate [113] trichlorethanol [113] and valicylate [80 113] and after intravenous injection of NAPA [80] following injection of NAIA for example the total recovery of NAPA metabolites in the

TABLE 8-3 URINARY EXCRETION OF N-ACETYL-p-AMINOPHENOL (NAIA) GLUCURONIDE AND TOTAL p-AMINOPHENOL IN THREE PATIENTS WITH CONGENITAL NONHEMOLYTIC JAUNDICE AFTER A SINGLE INTRAVENOUS INJECTION OF NAIA 10 MG PER KG

| Patient age and sex   | Patient             |                        | Controls of same age and sex |                        |
|-----------------------|---------------------|------------------------|------------------------------|------------------------|
|                       | NAPA<br>glucuronide | Total<br>p-aminophenol | NAPA<br>glucuronide          | Total<br>p-aminophenol |
| J d E (8 months male) | 8.9                 | 50                     | 25                           | 71                     |
| J D (8 years male)    | 16.6                | 48                     | 28                           | 58                     |
| R S (8 years female)  | 18.0                | 65                     | 30                           | 51                     |
|                       |                     |                        | 25                           | 55                     |
|                       |                     |                        | 29                           | 54                     |

Amounts excreted in 6 hr in per cent of dose administered

anatomy when bile aspirated from the gallbladder was found to contain 70 mg bilirubin per 100 ml about one third conjugated with glucuronic acid [80]. Fecal urobilinogen excretion in this 8-year-old girl was 16.9 mg per 24 hr. The lack of reliable data on fecal pigment excretion by healthy children of this age group makes it impossible at present to determine whether this represents a significant reduction in fecal urobilinogen excretion. It is of interest that in contrast to extrahepatic biliary obstruction the color of the feces of all four icteric children was normal [80].

In the original report of this syndrome [63] it was stated that fecal urobilin excretion was normal but these results were obtained by a method not in common use [111]. For instance in a 6-year-old boy originally reported as having normal urobilin excretion [63] fecal urobilinogen excretion was found to be 1.1 mg per 24 hr [80].

In all patients studied the serum was deeply icteric. Bilirubin concentrations ranged from 16 to 38 mg per 100 ml and practically all of it gave an indirect van den Bergh reaction. On paper chromatographic analysis no evidence for the presence of bilirubin glucuronide was obtained [10, 54] (Fig. 8-14). In some instances the plasma was extracted and bilirubin was prepared in crystalline form [63].

#### *Formation of Glucuronides in Vivo and in Vitro*

The above findings suggest that the metabolic defect in these patients involves the conversion of bilirubin to its water-soluble conjugates [54, 55]. Direct demonstration of such a disturbance *in vivo* is technically difficult but it was found that in addition to impaired formation of bilirubin glucuronide formation of other glucuronides is also reduced [54, 56]. This was demonstrated with a variety of compounds which ordinarily are excreted in part as glucuronides.

After oral administration of N-acetyl *p*-aminophenol (NAPA), the concentration of NAPA glucuronide in the plasma was much less in an icteric child than in a normal subject of the same age and sex [56] (Fig. 8-15). When tetrahydrocortisone or tetrahydrocortisol was given by rapid intravenous infusion the rate of disappearance of these compounds from the plasma of icteric children was slower than in normal subjects [112, 113] (Fig. 8-16). A difference in disappearance rate was not noted with cortisol<sup>2</sup> [112, 113] but the metabolites of cortisol conjugated with glucuronic acid appeared in the plasma at a slower rate than in normal subjects [112] (Fig. 8-17). Following infusion of cortisol C<sup>14</sup> recovery of labeled metabolites in the urine of icteric patients was quantitatively comparable to that in normal subjects but the fraction of glucuronic acid-conjugated metabolites was much smaller and the fraction of other conjugates larger than in normal individuals [112]. The findings indicate

<sup>2</sup> Cortisol in contrast to tetrahydrocortisol does not form a glucuronide because it lacks the 3 $\alpha$ -ol function in ring A [114].

formation of glucuronides is demonstrable with all test substances used but glucuronide synthesis is by no means absent. For bilirubin on the other hand a much more severe defect must be assumed if the pigment retention in the plasma and the impaired excretion of bilirubin in the

FREE AND GLUCURONIDE CONJUGATED STEROIDS  
 IN PLASMA FOLLOWING INFUSION OF HYDROCORTISONE- $4-C^{14}$

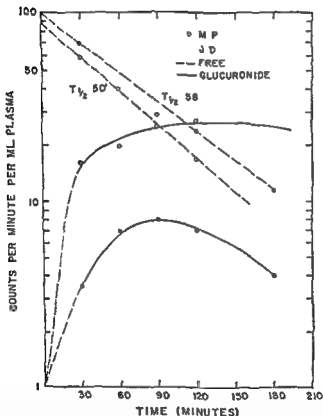


Fig 8-17 The rate of disappearance of injected hydrocortisone (cortisol) was similar in a child with congenital nonhemolytic jaundice (JD) and in a control subject of the same age and sex (MP). The rate of appearance of glucuronic acid-conjugated metabolites of cortisol in the plasma was slower in the icteric patient than in the control subject. (Courtesy of R. E. Peterson and R. Schmid unpublished observation)

bile and of urobilinogen in the feces are due solely to a disturbance in the conjugating apparatus. The problem is strikingly similar to that in the icteric rats discussed earlier in both the human and the murine syndromes it remains unexplained why the defect affects glucuronide forma

urine of jaundiced children was comparable to that in control subjects but the glucuronic acid conjugated fraction was reduced in the presence of icterus (Table 8.5).

Reduced formation of glucuronides has also been reported with salicylate [113] but interpretation of the results is complicated because

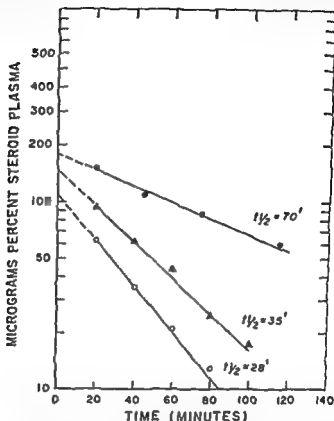


Fig. 8-16 Disappearance of tetrahydrocortisone (tetrahydrocortisol) from plasma following intravenous administration: ● patient with congenital nonhemolytic jaundice; ▲ patients (3) with parenchymal liver disease; ○ normal subjects (4). (By permission of L. F. Jefferson et al. [112].)

normal individuals excrete only 10 to 15 per cent of the administered dose as glucuronides and because salicylate forms both phenolic and acyl glucuronides [116]. In two icteric children given sodium salicylate by mouth the urinary excretion of salicyl glucuronides and the total excretion of salicylate metabolites was reduced [113]. In the glucuronide fraction, the reduction was more pronounced for the phenolic type of glucuronide than for the acyl type [113] which is surprising since bilirubin forms an acyl glucuronide [83].

These findings do not permit a final interpretation. A reduction in the

infancy with severe unexplained jaundice. These observations suggest that the defect is genetically determined and may be inherited as a Mendelian recessive trait. Unfortunately the available reports do not permit a calculation of the ratio of affected children to unaffected siblings.

In parents and nonaffected siblings of icteric patients serum bilirubin concentrations were consistently normal [106-107]. The magnitude of glucuronide formation *in vivo* was studied in parents and siblings of six icteric patients [80-113]. It should be noted that the significance of such

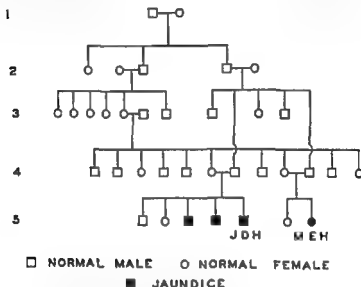


Fig 8-18 Pedigree of a family with congenital non-spherocytic jaundice. This family is part of an interrelated family group exhibiting a total of seven recognized cases of jaundice (By permission of R. G. Gellera et al [113]).

family studies is limited by the existing uncertainty with regard to the exact pathogenesis of the syndrome and by the fact that even in patients with jaundice glucuronide formation is only moderately reduced.

Childs et al [113] fed sodium salicylate to parents, adult siblings and grandparents of three icteric patients and estimated the excretion of acyl glucuronides and of other acylate metabolites in the urine. In the majority but not in all of the parents a slight reduction in excretion of glucuronides was demonstrable [113]. The mean value for acyl glucuronide excretion by parents, adult siblings and grandparents combined was moderately reduced in two families and questionably reduced in the third. These findings were believed to indicate that the abnormal gene is recessive with respect to bilirubin but incompletely dominant for other glucuronide-forming compounds [113].



tion with bilirubin more severely than with other glucuronide-forming compounds

Studies of liver tissue *in vitro* in this syndrome have not been reported except in two patients who originally were believed to have Gilbert's disease [116] but who probably had a defect similar to that under discussion [117]. In these two patients liver homogenate was found to lack the ability to form glucuronides both with bilirubin and with *o*-aminophenol [116] but in one instance the bile contained "direct reacting bilirubin" although chromatographic analysis of the pigment was not attempted [116].

In the author's laboratory liver tissue was studied from the 8 year-old girl (R.S.) who exhibited serum bilirubin levels of 16 to 20 mg per 100 ml. Bilirubin and *o*-aminophenol were incubated with liver homogenate and with a microsomal preparation respectively. It was observed that liver homogenate failed to form direct reacting bilirubin [90] but the microsomal preparation synthesized *o*-aminophenyl glucuronide at a rate which was about half of that found with similar preparations of male rat liver [78]. In addition, however, bile aspirated from the gall bladder contained 70 mg per 100 ml bilirubin of which about one-third was in the form of the glucuronide [118] and less than 5 per cent in the form of the alkali stable sulfate [77]. This observation must indicate that formation of bilirubin glucuronide *did* occur *in vivo* but with the available methods it could not be demonstrated *in vitro*. A definite explanation for this perplexing finding cannot be offered. The possibility should be considered, however, that the defect in glucuronide formation may be associated with other abnormalities in bilirubin metabolism perhaps involving the binding of pigment to plasma proteins or bilirubin transport into the liver cells.

The available evidence relating to the pathogenesis of congenital non-hemolytic jaundice may be summarized as follows: marked retention of unconjugated bilirubin occurs in the plasma and biliary excretion of bilirubin is reduced. This is the result of a disturbance in the liver involving conversion of bilirubin to its water soluble conjugates. The precise nature of this defect is not yet clear and it is not known whether it involves only reduced activity of the glucuronyl transferase system in the microsomes or includes other aspects of bilirubin metabolism in the liver.

### *Genetic Considerations*

The original description of this syndrome [53] indicated a definite familial occurrence. Six cases occurred in three related families and later an additional genetically related patient was discovered (Fig. 8-18) [106]. The two patients observed by Klingberg [109] were siblings and a double cousin of the patient reported by Rosenthal et al. [107] died in

examination [120]. The bilirubin level frequently shows considerable daily fluctuation. Among 58 patients followed for various lengths of time 13 had levels of 1 mg per 100 ml or less at least at some time during the observation period [120]. In this group the average of the highest values observed in each patient was 2.72 mg per 100 ml and the average of the lowest observed levels 1.67 mg per 100 ml [120]. In 81 per cent of the patients the initial concentration was less than 3 mg per 100 ml. Virtually all the serum bilirubin gives an indirect van den Bergh reaction [120] and on paper chromatographic analysis no conjugated bilirubin is detectable [80]. Bilirubinuria is absent and conventional liver function tests give normal results except for occasional increased Bromsulphalein retention [117, 125]. Liver and spleen are usually not enlarged [120, 126] and histologic examination of the liver reveals no significant abnormalities [120].

It is often stated that the hyperbilirubinemia is associated with subjective symptoms such as fatigue, asthenia, weariness and dyspepsia [121, 122, 126]. The multiplicity of complaints and the failure to elicit any common pattern of symptoms suggest that these manifestations may be unrelated to the disorder in pigment metabolism [120]. Indeed it seems likely that many of the symptoms develop *after* the patient has become aware of his icterus and are related to anxiety rather than to organic disease [120]. For example four young physicians exhibiting serum bilirubin levels of 1.4 to 2.8 mg per 100 ml denied any systemic symptoms whereas two young ex-service men whose mild hyperbilirubinemia was detected during active duty entitling them to disability payments complained of excessive fatigue and lassitude [80].

In some of the patients studies of erythrocyte survival with the Cr<sup>51</sup> method suggested a mild and compensated hemolytic state [120, 127]. It is doubtful whether this slight decrease in erythrocyte life span could account for the chronic hyperbilirubinemia since as discussed earlier the ability of the normal liver to remove bilirubin from the blood stream is much in excess of the pigment load presented by the physiologic decay of erythrocytes. The fecal excretion of urobilinogen is usually normal or may be slightly decreased.

The pathogenesis of this syndrome is not clear and there is much evidence to suggest that persistent mild hyperbilirubinemia may be the result of several unrelated disturbances. Hult [126] and Volwiler and Elliott [128] showed that chronic icterus may be a late manifestation of infectious hepatitis. On the other hand the finding of unexplained mild hyperbilirubinemia in several members of a family [120-123, 129] and the occurrence of mild icterus in three successive generations [130] suggest that the abnormality may be genetically controlled. Finally it should be considered that some of the individuals with an apparent increase in indirect reacting bilirubin concentration may simply represent

The parents of three other icteric children were studied by intravenous injection of NAPA followed by estimation of the excreted NAPA glucuronide and total aminophenol in the urine [50]. As seen in Table 8-6 four out of the six parents tested excreted at least as much NAPA glucuronide as control subjects of the same sex, while in two the values were slightly reduced. The findings which are at variance with those

TABLE 8-6 URINARY EXCRETION OF N-ACETYL-P-AMINOPHENYL (NAPA) GLUCURONIDE AND TOTAL P-AMINOPHENOL IN PARENTS OF PATIENTS WITH CONGENITAL NONHEMOLYTIC JAUNDICE AFTER A SINGLE INTRAVENOUS INJECTION OF NAPA 10 MG PER KG

| Parents          | NAPA<br>glucuronide | Total<br>p-aminophenol |
|------------------|---------------------|------------------------|
| J d F            |                     |                        |
| Mother           | 11                  | 61                     |
| Father           | 14                  | 54                     |
| J D              |                     |                        |
| Mother           | 20                  | 66                     |
| Father           | 23                  | 74                     |
| R S              |                     |                        |
| Mother           | 45                  | 81                     |
| Father           | 16                  | 70                     |
| Normal controls  |                     |                        |
| 3 females (mean) | 39                  | 69                     |
| 3 males (mean)   | 32                  | 56                     |

Amounts excreted in 6 hr in per cent of dose administered

reported by Childs et al [118] failed to reveal a consistent reduction in glucuronide formation in parents of patients with congenital non hemolytic jaundice. They do not support the concept of a dominant inheritance of this metabolic disturbance [118].

#### CONSTITUTIONAL HEPATIC DYSFUNCTION (GILBERT'S DISEASE)

Since the original report by Gilbert [119] patients with unexplained mild and chronic elevation of indirect reacting serum bilirubin have been reported with increasing frequency [120]. The condition has variously been referred to as icterus intermittens juvenilis [121], hereditary non hemolytic bilirubinemia [122], familial nonhemolytic jaundice [123], and constitutional hepatic dysfunction [124]. None of these terms is adequate but any is perhaps preferable to the frequently employed

Gilbert's disease since most of Gilbert's cases probably suffered from what is now recognized as a compensated hemolytic state.

Mild icterus may be detected shortly after birth or may be recognized only later in life [120, 121]. At times the patient is unaware of his abnormality until hyperbilirubinemia is detected on incidental laboratory

defect is probably genetically controlled and may be transmitted as a Mendelian dominant [123, 135]

Finally, this syndrome must be considered in relation to the severe congenital and familial nonhemolytic jaundice (Crigler Najjar syndrome). Although clinically the two conditions differ mainly in the degree of icterus a clear distinction is not always possible. Indeed it has been suggested that the two syndromes may represent different degrees of the same defect [120-136]. Against this concept is the finding that patients with constitutional hepatic dysfunction form glucuronides at a normal

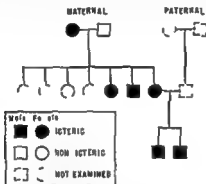


Fig 8-19 Pedigree of a family with constitutional hepatic dysfunction (Gilbert's disease) (By permission of W G Baroody et al [140])

rate and that parents and siblings of patients with congenital nonhemolytic jaundice fail to exhibit mild hyperbilirubinemia. This renders it unlikely that constitutional hepatic dysfunction represents the heterozygous form of congenital nonhemolytic jaundice but suggests that the two syndromes are genetically and pathogenetically different.

## ICTERUS WITH INCREASED LEVELS OF CONJUGATED BILIRUBIN

### CHRONIC IDIOPATHIC JAUNDICE (DUBIN JOHNSON SYNDROME)

In 1934 Dubin and Johnson [137] and Sprinz and Nelson [138] described a hitherto unrecognized syndrome characterized by chronic icterus and the presence of an unidentified pigment in liver cells. Since the original description numerous cases of a similar nature have been observed and reported [139].

The presence of mild and often asymptomatic icterus may be detected in childhood or during adult life [139]. Hyperbilirubinemia is usually light in degree and exhibits marked fluctuation in intensity [138-139]. Characteristically the plasma contains a significant fraction of bilirubin which gives a direct van den Bergh reaction [138-140] and conjugated

those variants which lie outside the statistical confidence limits of the range of normal bilirubin concentrations and thus do not constitute a true abnormality.

The finding that the pigment retained in the plasma is always unconjugated bilirubin [80] suggests that the primary defect lies in the conversion of bilirubin to its conjugates. As discussed earlier such defects may involve the binding of bilirubin to plasma proteins, its transport across the liver cell membrane, the structure of the microsomal particles, or the activity of the conjugating enzyme system. It has been claimed that in some of these patients a reduction in glucuronyl transferase activity can be demonstrated in liver tissue obtained by needle biopsy [131] but details of the technique employed have not been published. On the other hand it was found that *in vivo* such patients are able to form and excrete glucuronides at a rate comparable to that of nonicteric individuals [117, 120, 132] (Table 8-7). This finding suggests that the glucuronide-forming mechanism can not be significantly impaired and that the hyperbilirubinemia must be due to other defects [117, 132].

TABLE 8-7 URINARY EXCRETION OF N-ACETYL-P-AMINOPHENYL (NAP) (GLUCURONIDE AND TOTAL P-AMINOPHENOL IN FIVE MALE PATIENTS WITH CONSTITUTIONAL HEPATIC DYSFUNCTION AFTER A SINGLE INTRAVENOUS INJECTION OF NAP 10 MG PER KG

| Patient        | Serum bilirubin<br>(total mg/100 ml) | NAP<br>glucuronide | Total<br>p-aminophenol |
|----------------|--------------------------------------|--------------------|------------------------|
| DDI            | 2.9                                  | 27                 | 60                     |
| TC             | 1.5                                  | 30                 | 58                     |
| CS             | 1.6                                  | 23                 | 62                     |
| AD             | 2.1                                  | 46                 | 75                     |
| JM             | 2.8                                  | 39                 | 78                     |
| 6 normal males | 0.6<br>(0.4-0.9)                     | 32<br>(20-36)      | 61<br>(41-74)          |

Amounts excreted in 6 hr in per cent of dose administered

The occurrence of unexplained hyperbilirubinemia in more than one member of a family has repeatedly been reported [120-123, 129, 130, 133-135]. Dameshek and Singer [123] described 2 families with 3 and 7 icteric members and among 58 patients with unexplained hyperbilirubinemia studied by Foulk et al [120] 11 had a positive family history for jaundice. In the group of 15 patients reported by Alwall et al [135] 55 per cent of the siblings and 26 per cent of the probands' parents had bilirubin levels above 1.3 mg per 100 ml. The pedigree of a family studied by Baroody et al [129] is reproduced in Fig. 8-19. These findings suggest that in some instances of mild chronic hyperbilirubinemia the

defect is probably genetically controlled and may be transmitted as a Mendelian dominant [123-135]

Finally this syndrome must be considered in relation to the severe congenital and familial nonhemolytic jaundice (Crigler-Najjar syndrome). Although clinically the two conditions differ mainly in the degree of icterus a clear distinction is not always possible. Indeed it has been suggested that the two syndromes may represent different degrees of the same defect [120-136]. Against this concept is the finding that patients with constitutional hepatic dysfunction form glucuronides at a normal

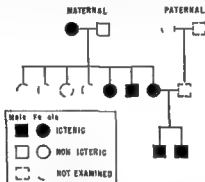


Fig 8-19 Pedigree of a family with constitutional hepatic dysfunction (Gilbert's disease) (By permission of W. G. Baroddy et al [19])

rate and that parents and siblings of patients with congenital nonhemolytic jaundice fail to exhibit mild hyperbilirubinemia. This renders it unlikely that constitutional hepatic dysfunction represents the heterozygous form of congenital nonhemolytic jaundice but suggests that the two syndromes are genetically and pathogenetically different.

## ICTERUS WITH INCREASED LEVELS OF CONJUGATED BILIRUBIN

### CHRONIC IDIOPATHIC JAUNDICE (DUBIN-Johnson SYNDROME)

In 1954 Dubin and Johnson [137] and Sprinz and Nelson [138] described a hitherto unrecognized syndrome characterized by chronic icterus and the presence of an unidentified pigment in liver cells. Since the original description numerous cases of a similar nature have been observed and reported [139].

The presence of mild and often asymptomatic icterus may be detected in childhood or during adult life [139]. Hyperbilirubinemia is usually slight in degree and exhibits marked fluctuation in intensity [138-139]. Characteristically the plasma contains a significant fraction of bilirubin which gives a direct van den Bergh reaction [138-140] and conjugated

bilirubin can be demonstrated on paper chromatography [80] In the majority of patients bile pigment is detectable in the urine [139 140]

The hyperbilirubinemia may be entirely asymptomatic or may be associated with weakness increased fatigability and protean gastrointestinal manifestations [139] Enlargement and tenderness of the liver are occasionally observed [139] The results of conventional liver function

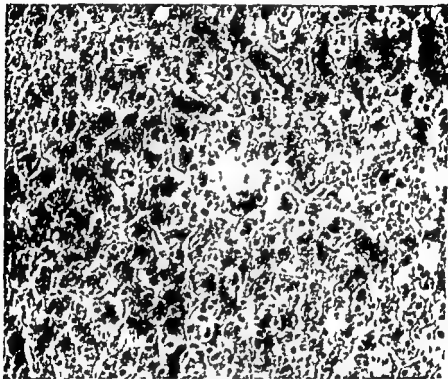


Fig. 8-20 Liver in chronic idiopathic jaundice (Dubin-Johnson syndrome) Pigmentation is particularly marked in parenchymal liver cells of the centrilobular area (By permission of I. N. Dubin et al [137])

tests may be either normal or moderately impaired [139 140] In the majority of patients some increase in Bromsulphalein retention is found but it is not known whether this is due to retention in the plasma of unaltered dye or to accumulation of Bromsulphalein metabolites Oral cholecystography frequently fails to display the gallbladder [139] or visualization is only faint [140] Intravenous administration of contrast medium may yield a higher percentage of visualization [139]

Except for the presence of dark pigmentation the gross appearance of the liver is usually normal [139] The tissue core obtained by needle biopsy is often described as green black or dark gray [139 140] On histologic examination of the liver a slight degree of fibrosis or fatty

infiltration may be detectable [139] but the most striking finding is the presence of intracellular coarsely granular brown pigment which is particularly conspicuous in cells of the centrilobular area (Fig 8-20). The pigment tends to be confined to parenchymal cells [138] and to a lesser degree to Kupffer cells [139-140]. The nature of this pigment is not known. Histochemically it differs from bile pigment, hemosiderin, hematin, and melanin [138-139]. It has been suggested that it may belong to the ill-defined group of lipofuscins [137, 138-141-142] but in more recent studies this has been disputed [140].

Of importance is the report by Popper's group [140] that in instances of familial occurrence of this syndrome wide variations in the degree of pigmentation may be observed in the liver of different patients. In two

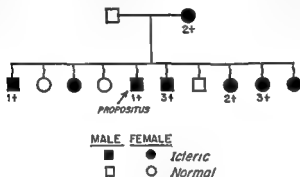


Fig 8-21 Pedigree of a family with chronic idiopathic jaundice. The degree of hepatic pigmentation is indicated on an arbitrary scale of 1+ to 3+. In two patients liver biopsies were not performed. (By permission of R. L. Wolf et al. [140].)

families with 10 icteric members, histologic examination of the liver revealed two instances with large amounts of pigment, four in which the degree of pigmentation was slight, and two with only minimal pigment deposition. In the last group the character and quantity of the pigment differed little from the centrilobular pigment often found in parenchymal cells of normal livers [143]. The observations are believed to indicate that the accumulation of pigment may not be due to a specific abnormality but may simply represent faulty excretion of a pigment which normally is formed in the liver and excreted in the bile [140]. The postulated abnormality would include excretion of conjugated bilirubin [139], Bromsulphalein [138-140], rose bengal [140], contrast media employed for cholecystography [139], and the unidentified pigment present in the liver.

The syndrome occurs in both sexes and has been observed in widely varying ethnic groups including Negroes, Persian Jews, and Caucasians [139]. A positive family history of icterus is obtained in a significant number of patients [139], but a definite familial incidence can be estab-



lished only by systemic performance of liver biopsies on family group [140]. The pedigree of a Puerto Rican family studied by Wolf et al [140] is given in Fig. 8-21. One parent and seven out of ten siblings exhibited hyperbilirubinemia. Liver biopsies performed in all but two of these patients revealed a significant but variable degree of pigmentation. This and similar observations [144-146] suggest that the defect may be genetically controlled but additional studies are needed to clarify the exact mode of inheritance.

Clinically, chronic idiopathic jaundice has many similarities to constitutional hepatic dysfunction. Both disorders are characterized by familial incidence, chronic and fluctuating hyperbilirubinemia, and a benign course. In chronic idiopathic jaundice, however, the presence of an excretory defect and the accumulation of pigment in the liver permit a clear separation from constitutional hepatic dysfunction in which the defect appears to be limited to the conversion of bilirubin to its conjugate. In the latter condition the plasma contains only nonconjugated bilirubin and bilirubinuria is absent, whereas in chronic idiopathic jaundice conjugated bilirubin is present in plasma and urine.

#### OTHER FORMS OF CHRONIC HYPERBILIRUBINEMIA WITH CONJUGATED BILIRUBIN

In 1948 Rotor and his associates [147] described a Philippine family with several icteric members, three of whom were studied in detail. Mild and fluctuating icterus was discovered shortly after birth or during childhood and persisted throughout life. Serum bilirubin concentrations were slightly to moderately increased and the van den Bergh reaction was predominantly direct, but bilirubinuria was not noted. There was intermittent epigastric discomfort with occasional frank abdominal pain and fever. There was nothing to suggest cholelithiasis or increased hemolysis. Cholecystography and estimation of erythrocyte life span or fecal urobilinogen excretion were not reported. The only significant abnormality was marked retention of Bromsulphalein. Liver biopsy performed in one patient showed no significant histologic abnormalities.

A pedigree of this family indicates the presence of jaundice in a total of eight members in three generations (Fig. 8-22). An additional two members gave a history of jaundice although at the time of examination they were not icteric. Unfortunately, only three patients were reported in detail [147-148].

Findings similar to those of Rotor et al [147] were reported from Italy where several members of two families were found to have mild chronic elevation of direct reacting serum bilirubin [149-151]. Another case probably representing a similar syndrome was recently reported [152]. A 29-year-old Japanese woman had unexplained asymptomatic icterus with bilirubinuria for at least 11 years. Extensive studies revealed the follow-

ing the serum bilirubin levels fluctuated from 4.0 to 7.6 mg per 100 ml with approximately half of the pigment present in conjugated form. Conventional liver function tests including oral cholecystography failed to reveal abnormal results except for marked retention of Bromsulphalein and a positive cephalin flocculation test. Hepatic tissue obtained on two separate occasions showed no histologic abnormalities and pigmentation was absent.

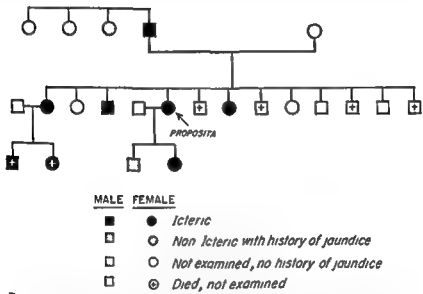


Fig. 8-2. Pedigree of a family with chronic hyperbilirubinemia with conjugated bilirubin. Liver biopsy was performed in only one patient showing no significant histologic abnormalities (Bjorkman and Eklund [1958]).

An older sister of this patient who also has long-standing and unexplained icterus had a cholecystectomy 10 years ago. Gallstones were present but postoperatively the jaundice persisted without improvement. In addition to hyperbilirubinemia the patient also has marked retention of Bromsulphalein.

All the patients exhibit findings which suggest a defect in hepatic excretory function. The occurrence of icterus in several members of a family may indicate that the disturbance is genetically controlled but the available data are inadequate to establish a definite genetic pattern. The absence of pigment in liver cells and the marked abnormality in clearance of Bromsulphalein suggest that the syndrome may differ from chronic idiopathic jaundice but this argument loses force with the recent demonstration that the degree of hepatic pigmentation may be quite variable or indeed minimal in the latter syndrome [140]. If more extensive studies confirm these findings it may be difficult or impossible to make a clear

distinction between chronic idiopathic jaundice and the syndrome described in the Philippine, Japanese and Italian families. A definite classification of the various forms of chronic icterus associated with an hepatic excretory defect must await a better understanding of the fundamental abnormalities involved.

## SUMMARY

1 Several syndromes with chronic nonhemolytic icterus have been shown or are suspected to be due to a genetically controlled abnormality in the liver. These can be divided into two major groups depending on whether the principal defect involves conversion of bilirubin to its conjugates or excretion of conjugated pigment in the bile. The first group comprises patients with congenital familial nonhemolytic jaundice (Crigler-Najjar syndrome) and with constitutional hepatic dysfunction (Gilbert's disease). The second group includes chronic idiopathic jaundice (Dubin-Johnson syndrome) and related syndromes. (Congenital abnormalities in the development of intrahepatic bile ducts are not discussed here.)

2 *Congenital nonhemolytic jaundice* is a rare familial and probably recessively inherited disorder characterized by high concentrations of unconjugated bilirubin in the plasma and reduced pigment excretion in bile and feces. Disturbances of the central nervous system resembling kernicterus are frequently present. Defects in the glucuronide-forming mechanism have been demonstrated *in vivo* and *in vitro* but it is not established with certainty whether the severe hyperbilirubinemia is due solely to reduced function of the conjugating enzyme system or to additional and as yet unknown abnormalities.

3 Patients with *constitutional hepatic dysfunction* usually exhibit a much milder degree of hyperbilirubinemia. The concentration of unconjugated bilirubin in the serum fluctuates and bilirubinuria is absent. Mild and intermittent icterus may be the only manifestation or it may be associated with lassitude, increased fatigability, and gastrointestinal complaints. There is no convincing evidence to suggest that the activity of the glucuronide-forming enzyme system in the liver is reduced and the cause of the hyperbilirubinemia is not yet clear. The syndrome frequently occurs in several members of a family suggesting that the metabolic defect may be genetically controlled. It is not known whether the same abnormality is present in all cases observed or whether a similar disturbance may be acquired, e.g. in patients who have had infectious hepatitis.

4 *Chronic idiopathic jaundice* is a syndrome of possible hereditary nature, primarily involving hepatic excretory function. The plasma contains increased concentration of conjugated bilirubin. Bilirubinuria is frequently observed and the liver often exhibits intracellular deposition

of an unidentified pigment. A few instances of a similar syndrome but lacking pigmentation of the liver have been described in families of Philippine, Italian and Japanese origin. The relationship of these cases to chronic idiopathic jaundice is not clear.

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### **Part Three**

#### **Diseases Primarily Related to Disorders of Amino Acid Metabolism**

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## Chapter 9

### Familial Goiter\*

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*John B. Stanbury*

This chapter is concerned with the metabolic aspects of familial goiter. The patients to be discussed have, with few exceptions, retarded mental or skeletal development and, if untreated with thyroid substance, show the outward appearance of thyroid hormone deficiency. Goiter may be present at birth but more commonly becomes noticeable during childhood. Siblings may be similarly afflicted. These patients are nosologically distinguished from clinically similar patients who have endemic goiter. Several reviews have appeared [1-6] since the first cases were reported by Osler in 1897 [7].

The results of laboratory investigations of many subjects with familial goiter and hypothyroidism have made it possible to classify some of them into categories according to specific and identifiable biochemical lesions. In each category the lesion is specific and distinct, but the net effect is the same: synthesis and delivery of thyroid hormone are inadequate. Before considering these groups in turn it is appropriate to survey briefly the principal metabolic pathways of iodine in the body. These pathways are illustrated in Fig. 9.1. Competent reviews are available elsewhere [8-12].

### METABOLISM OF IODINE

#### ABSORPTION AND DISTRIBUTION

Iodide<sup>1</sup> is absorbed through the gastrointestinal tract as inorganic iodide and is rapidly distributed throughout the extracellular fluid of the body. Free iodine and the iodine of most organic compounds is reduced

<sup>1</sup>This work was supported in part by grant from the Public Health Service (A-1880).

In this chapter the term *iodine* is used in a generic sense to encompass all forms and oxidation states unless otherwise indicated. Similarly the term *labeled iodine* is used generically to indicate the iodine which is marked by a radioactive isotope of iodine. The term *iodide* always indicates the reduced ionic form of the element.



normally lies between 10 and 35 ml per min. The small quantities of iodide removed from the plasma by the salivary and gastric glands are returned to the plasma after absorption in the small bowel. Small amounts of iodide are removed by the mammary glands during lactation. Losses of inorganic iodide in feces, sweat, and expired air are small.

### IODIDE TRAPPING

Iodide cleared from the plasma by the thyroid gland is retained momentarily in the gland as diffusible and dialyzable inorganic iodide. Only 1 to 2 per cent of the iodine in the gland fails to precipitate upon treatment with trichloroacetic acid. This small amount is in much higher concentration than is the iodide of the plasma. In spite of the concentration gradient, the inorganic iodide of the gland is in free and ready exchange with the plasma iodide. Thus if the inorganic iodide of the gland is labeled with  $I^{131}$ , the latter can be diluted out of the gland almost instantaneously by administration of a large amount of unlabeled iodide.

Iodide trapping is oxygen dependent and inhibited by cyanide, azide, and a number of other substances which interfere with oxidative processes in cells, as well as by sulfhydryl inhibitors [14, 15]. The mechanism of transport across the cell wall against a concentration gradient is entirely unknown. Trapping requires an expenditure of energy and is doubtless enzymatically controlled, as are probably most other biochemical processes in the gland. Oxidative phosphorylation is involved in the trapping of iodide [15].

The normal resting thyroid maintains a concentration of iodide against the plasma of approximately 20:1. This may be increased to several hundred to one in the hyperplastic gland. The magnitude of the thyroid/plasma ratio is determined by the thyrotropic hormone, but the ratio is also governed by the quantity of stored iodine within the gland itself [16, 17]. The ratio is constant over wide plasma concentration ranges of iodide, but with increasing concentration the ratio eventually falls toward unity as the holding capacity of the gland approaches saturation.

Iodide trapping has been studied in model systems on the presumption that the iodide is held in simple anionic electrostatic binding to intracellular (or cell surface) proteins. Homogenate of thyroid tissue within a semipermeable bag concentrated iodine from the surrounding bath [18, 19], but there was no excess in the bag as inorganic iodide. The accumulated iodine was found in part as monoiodotyrosine (MIT) and in part as an unidentified component which was extractable slowly into carbon disulfide and accordingly was also in an oxidized form. Accumulation was blocked by reducing agents and by heat inactivation, but not by perchlorate. Inhibition by catalase indicated that a peroxidase is involved in the process. Thus the model system failed to imitate the *in vivo* iodide trap but suggested the existence of an intermediate bound form of oxidized

iodine This might conceivably be a storage form either firmly ad orb ed or in molecular linkage

Thiocyanate ( $\text{SCN}^-$ ) and perchlorate ( $\text{ClO}_4^-$ ) ions prevent iodide trapping [20, 21] perchlorate is about ten times as potent as thiocyanate ✓ If either of these substances is administered after trapping of iodide has occurred trapped iodide is rapidly discharged from the gland but iodine which has been chemically bound to iodinated tyrosines or thyronines is retained Chronic administration of  $\text{SCN}^-$  may lead to the development of goiter as was observed in certain patients treated with this substance for hypertension It is not known whether  $\text{SCN}^-$  and  $\text{ClO}_4^-$  block trapping by interfering with cell membrane transport or by competing for binding sites within the gland Preferential concentration of these ions by the thyroid has not been demonstrated with certainty [22-23]

#### OXIDATION AND ORGANIFICATION OF IODIDE

The next step after iodide trapping in hormone synthesis is the oxidation of iodide and its displacement of a hydrogen from the 3 position of tyrosyl residues The tyrosine is thought to be in peptide linkage because organically bound iodine is precipitated with protein precipitants such as trichloroacetic acid The degree of complexity of this step is undetermined There may simply be an oxidation of iodide with iodination of tyrosyl residues proceeding *pari passu* or there may be an intermediate storage form of iodine which requires a transferring enzyme in order to gain access to tyrosine Free or peptide-linked tyrosine is iodinated by iodine nonenzymatically *in vitro* but it is entirely possible that the process is rate-governed enzymatically in the gland

The nature of iodide oxidation in the thyroid has been a particularly baffling problem Iodide is oxidized both by slices *in vitro* and by cell free homogenates [19-24-26] and this oxidation is abolished by heat Inhibition of iodide oxidation in thyroid slices by cyanide, azide sulfide and carbon monoxide was interpreted by Schachner et al [26] as indicating that cytochrome oxidase is involved but they could not eliminate the possibility of participation by a peroxidase ✓

There seems to be good evidence that peroxidase is concerned in iodide oxidation Not only has peroxidase activity been demonstrated many times in thyroid tissue [27-29] but also the oxidation of iodide which proceeds rather specifically in thyroid tissue is inhibited by catalase [19-30] Further a variety of substances which inhibit organification of iodide *in vivo* or by thyroid slices such as thiourea and resorcinol either inhibit the peroxide-peroxidase system or act as competitive substrates for it [31-32] Finally there is a small but nonetheless necessary oxygen requirement [26] There seems little merit in the counterargument that a peroxidase has not been extracted from the thyroid because the requirement for oxidizing the minute amounts of iodide needed is very small

indeed. The inhibition by catalase indicates at least that peroxide is formed in the system. For a discussion of *in vivo* generation of peroxide see Chap. 46.

It has been suggested that the only requirements for iodide oxidation are large amounts of iodide and oxidizing conditions [11] and by implication that there may be no specific iodide oxidizing system in the thyroid gland. The colloid has a high oxidation-reduction potential and this is lowered by substances which have antithyroid activity such as thiouracil [27]. The possibility remains that a specific electron transfer system exists for converting iodide to iodine. Recently this possibility has been reopened by Alexander [33] who has described a specific iodide peroxidase which catalyzes the coupled peroxidation of iodide and is present in thyroid and salivary gland homogenates. The enzyme is a tissue component rather than a contaminant from the hemoproteins of the blood. Its action is inhibited by cyanide and thiourea but not by perchlorate.

The tyrosine iodinase system of Fawcett and Kirkwood [34] requires added tyrosine and cupric ion as the oxidant. Since the organification of iodide fails to take place when cupric ion is added to boiled tissue [19] one cannot consider this ion as the normal specific oxidant of iodide. More recently Serif and Kirkwood [30] have described a second system containing particulates from thyroid homogenates which oxidized iodide. This system is identical with or very similar in its properties to their earlier soluble system. The requirement for cupric ion is at variance with results obtained by others [19, 24, 35] although all have reported enhancement of oxidation and binding with copper.

Whatever the mechanism of iodide oxidation and transfer to tyrosyl residues may be, homogenates of thyroid tissue readily produce MIT and probably small amounts of diiodotyrosine (DIT) as well [34-36]. In addition, two iodinated compounds of unknown structure and significance appear [37]. Formation of all these compounds is much enhanced by addition of flavine nucleotides to the system [38] but an effect of pyridine nucleotides is not established [35, 36]. DPN appears to stimulate the production of still another unidentified iodinated component [38]. The MIT formed in homogenate systems is in peptide linkage and is released as the free amino acid by proteolytic hydrolysis. The poor yield of DIT has suggested that a different iodinating system is required for the iodination of MIT than for MIT formation [39].

#### COUPLING REACTION

The iodothyronines  $T_3$  and  $T_4$  are probably formed by coupling of two molecules of iodotyrosine and extrusion of a side chain [40]. There is little to support the alternative hypothesis that the iodothyronines are formed by iodination of thyronine. This amino acid has never been found in thyroid tissue [11]. Coupling is thought to occur while the respective



amino acids are in peptide linkage rather than in free form. The condensation proceeds slowly *in vitro* when only DIT is present in slightly alkaline solution [41] and is accelerated when a source of oxygen is present or when the carboxyl or amino group or both are covered in peptide linkage [42-43]. The reaction may be catalyzed by iodine [42]. Interestingly, coupling of *N*-acetylhistidyltyrosine is inhibited by thiourea [43].

The reaction may depend upon the production of free iodine within the gland as described above or a more specific mechanism may be involved. It has been proposed that condensation first involves oxidative formation of a quinoid intermediate which becomes a free radical which then unites with a second iodotyrosine molecule to form an iodothyronine and to split off pyruvic acid and ammonia [43].

No enzyme which promotes iodotyrosine coupling has been described. Failure of coupling to proceed in cell-free homogenate systems whereas  $T_4$  forms fairly readily in slices incubated with  $I^{131}$  strongly suggests that  $T_4$  formation *in vivo* either is enzymatically controlled or requires a high degree of cellular organization. Addition of a wide variety of nucleotides and other cofactors to homogenates has failed to promote the formation of  $T_3$  or  $T_4$  [24, 35, 36, 45].

Both  $T_3$  and  $T_4$  are formed in the thyroid gland.  $T_3$  could be formed either by deiodination of  $T_4$  or by the coupling of one molecule of MIT with one of DIT [42]. Roche and his colleagues favor coupling of one molecule of DIT and MIT since there was no prior incorporation of  $I^{131}$  into either  $T_3$  or  $T_4$  and since no  $T_4$  deiodinase has been detected in thyroid tissue. Roche et al. have also reported two other products of coupling in the thyroid, i.e., 3,3'-diiodothyronine and 3,5'-triiodothyronine [46, 47]. The presence of these two compounds in the thyroid or blood has not been confirmed and there is indirect evidence against their appearance in the blood at least in significant amounts [48, 49].

#### HORMONE STORAGE AND RELEASE

Thyroxine ( $T_4$ ) and 3,5,3'-triiodothyronine ( $T_3$ ) are retained in the thyroid gland in storage form within the colloid as peptide-linked residues within the specific thyroid protein thyroglobulin. Thyroglobulin is a glycoprotein which has a molecular weight of approximately 600,000 and an isoelectric point of 4.6 and which salts out of phosphate buffer between 1.4 and 1.75 *M* concentration.

Thyroglobulin is the principal iodinated component of the thyroid but at least two other iodinated components are present in much smaller concentration [50, 51]. One of these is associated with the nuclear fraction of homogenate of thyroid tissue; the other is soluble but has a slower sedimentation constant than thyroglobulin. Their role in thyroid function is not yet apparent. Ingbar et al. [52] have evidence from ion exchange

chromatography that preparations of sheep thyroglobulin made by salting-out techniques are not homogeneous. The same is true of human thyroglobulin when subjected to electrophoresis on hydrolyzed starch but the labeled iodine is almost entirely confined to a single sharp band which is the major protein component [53].

One of the principal unsolved problems of the thyroid gland is the nature of thyroglobulin with respect to the iodinated amino acids. There is every reason to suspect that thyroglobulin may contain widely varying amounts of these amino acids and yet such variability in amino acid content and sequence is not in keeping with current concepts of the specificity of protein structure. It may be that amino acid sequence is fixed and that degree of iodination can vary or that the iodinated amino acids are at N terminal or C terminal positions and can vary without altering the backbone of the thyroglobulin molecule. Alternatively it may be that thyroglobulin is generated as a highly specific structure closely dependent upon its template and that heterogeneity is introduced by breaking and remaking peptide chains in the course of the coupling reaction. Roche et al [54] found that some of the T<sub>4</sub>, DIT and tyrosine occupy N terminal positions of pork thyroglobulin. Most of the DIT and T<sub>4</sub> were internal. Glutamic acid, serine, lysine, alanine, leucine and small amounts of threonine and valine were also found at N terminal positions. T<sub>3</sub> and MIT were not found at N terminal positions. Pancreatin releases MIT first followed by DIT from thyroglobulin. T<sub>3</sub> and T<sub>4</sub> came off more slowly [55]. Little else is known of the fine structure of thyroglobulin.

The strongly stimulated thyroid gland may possibly secrete newly formed thyroid hormones directly into the blood without going through a thyroglobulin stage [56] but under normal circumstances T<sub>4</sub> and T<sub>3</sub> are released as needed from the thyroglobulin. Degradation of thyroglobulin is brought about through a sequence of lytic enzymes [57-60] which in turn may be activated by the thyrotropic hormone [61]. The protease and peptidase which have been extracted from the thyroid lack specificity for thyroglobulin. The protease has a pH optimum of 3.5 and is much more effective against hemoglobin than thyroglobulin. The peptidase has a pH optimum near neutrality.

Iodotyrosine releases T<sub>4</sub> and T<sub>3</sub> for diffusion into the blood. MIT and DIT also present in the thyroglobulin are also released but are deiodinated by a potent deiodinase which is present in the thyroid parenchymal cells [62-64]. This deiodinase is a microsomal enzyme which requires TPNH as a cofactor [65-66]. The removal of the first iodine from DIT occurs less rapidly than does the deiodination of MIT. The end products of the reaction are iodide and tyrosine. The freed iodide presumably is conserved for reutilization by the parenchymal cells. Iodotyrosine deiodinase has also been demonstrated in liver, kidney and other tissue. The presence of this enzyme in the thyroid cells prevents

the appearance of MIT and DIT in the peripheral blood. What purpose if any is served by this enzyme in other organs than the thyroid is not apparent. In an assay for this enzyme in 39 glands from patients with familial goiter Carr et al. found that the  $\Lambda_m$  was normal except in the specimens from eight cancers of the thyroid where the  $\Lambda_m$  was found to be low [66a].

#### HORMONE TRANSPORT AND FATE

Thyroxine ( $T_4$ ) is carried in the plasma in association with a carrier protein which has an electrophoretic mobility close to that of  $\alpha_2$ -globulin [67-69]. In addition a small amount of  $T_4$  is carried with albumin and recently a component has also been found in the prealbumin region in electrophoretograms of *in vitro* labeled plasma on hydrolyzed starch gel [70] and in Tris-maleate buffer [71]. There is some question as to whether the inter  $\alpha$  carrier protein and the prealbumin carrier may be one and the same and whether noted differences may be due to experimental conditions.  $T_3$  is also carried by the thyroid binding protein but it is less tightly bound and is found also in association with the other plasma proteins [72].

The equilibrium between free and bound iodothyronines may determine the amount of hormone which actually impinges upon cells to give the characteristic metabolic stimulating effect [73]. The amount of effective or free hormone is a function of the amount of hormone in the blood and the amount of carrier protein.

The thyroid hormones are degraded in the peripheral tissues through several pathways. Small amounts appear as glucuronides in the bile [73]. Isselbacher has found that these are formed by a liver microsomal enzyme which requires a uridine nucleotide [74]. Deamination and decarboxylation products have been demonstrated in brain tissue [70] and Albright et al. [76-77] have shown that the conversion of  $T_4$  to  $T_3$  may take place in kidney slices *in vitro*. More recently they have also observed the conversion in isolated cell particulate preparations of liver, kidney and other tissues [78].  $T_3$  fails to appear in the serum in significant amounts after intravenous administration of  $T_4$ . Several other investigators have described slow deiodination of  $T_4$  by liver homogenates [79-82]. Curiously enough rapid deiodination occurs in cell free and mitochondria free suspensions of liver tissue which have been heated to 100°C before addition of substrate [83]. This degradation has been traced to a heat stable microsomal enzyme which requires oxygen and  $Fe^{++}$  and is activated by cysteine and certain other organic reducing substances. Whether this enzymatic system has any function *in vivo* remains to be seen. Iodide released by degradation of iodothyronines is available for reentry into the iodide pool of the extracellular fluids. Little if any iodothyronine appears in the urine and only small amounts in the feces.

## PERIPHERAL ACTION OF THYROID HORMONES

It is not possible within the scope of this brief survey to examine in any detail the various theories which have been proposed to account for the stimulating effect of thyroid hormones on the oxygen consumption of cells. An attempt will be made only to mention the leading directions of investigation and to cite a few introductory papers from the profuse literature on this complicated subject. Only a few important contributions have been made since the excellent review of Wolff and Goldberg [11].

The peripheral action of thyromimetic substances is intimately related to structure but minimal structural requirements are not entirely determined. An ether bridge between the two rings is necessary but can be replaced with a thioether. The *p*-hydroxyl grouping can be replaced by a methoxy group and the alanine side chain can be radically altered. Various substitutions of nitro, amino, allyl, halogen and hydroxyl groups can be made for the iodines but almost always with diminution of activity [84-85]. Removal of one iodine from the 3' position enhances activity [80]. The relationships of structure to function are further complicated by the fact that a structural alteration may have differing metabolic effects depending on which of several assays is employed. This is not surprising when one considers that a structural change may alter absorption, protein binding, cell penetrance or degradation of the compound and that different assays may measure different effects of the substances in question.

The iodothyronine which ultimately affects the machinery of the cell has not been identified. The *in vivo* latency of several hours in the effect of thyroxine has suggested that it undergoes a structural alteration before it is active. The shorter latency of  $T_2$  and the still shorter one of the acetic acid analogue of  $T_2$  indicate a possible pathway of metabolism toward the effective substance.

**Effects on Peripheral Enzyme Activity.** A large number of cellular enzymes of various tissues are influenced by thyroid administration [87] or thyrotoxicosis [87a]. Succinic oxidase, amino acid oxidases, carbonic anhydrase, ascorbic oxidase, cytochrome oxidase, arginase, muscle hexokinase, amylase and others vary with the thyroid dosage. Lactic acid dehydrogenase and plasma pseudocholinesterase vary inversely. Some of these effects may be the result of binding of metals by iodothyronines. Thyroxine complexes with  $Cu^{++}$ ,  $Co^{++}$ ,  $Zn^{++}$ ,  $Mg^{++}$ ,  $Fe^{++}$  and  $Mn^{++}$ .

Thyroxine and several analogues inhibit a number of  $DI\Delta$ -dependent dehydrogenases. Among these are malic, glutamic, lactic, triosephosphate, yeast alcohol and yeast glucose-6-P-O<sub>2</sub> dehydrogenases [88]. All these enzymes contain zinc. With the possible exception of malic dehydrogenase, the effective concentration for  $T_4$  effect is above the physiologic

range by two or three orders of magnitude. The inhibition of malic dehydrogenase accounts for the apparent stimulation of succinic oxidation by  $T_4$ , since succinic dehydrogenase is inhibited by oxalacetate which is formed by the malic dehydrogenase. This action of L-thyroxine on dehydrogenases is shared by the dextrorotatory isomer and by other substances which do not have thyroxine-like activity [89-90]. Inhibition of DPN linked dehydrogenases has been attributed to a deleterious effect on mitochondria (which swell under the influence of thyroxine [91]), which permits leakage of DPN from these structures [92]. This may be one way in which dehydrogenase activity is inhibited but Wolff and Wolff found the inhibition in a system which was free of particulate material [88].

Thyroxine binding of  $Mg^{++}$  ion has been of particular interest because of the similarity of some of the effects of experimental magnesium deficiency to human thyrotoxicosis. Gershowitz et al. [93] have found that the inhibition of growth of rats caused by administration of large amounts of  $T_4$  can be at least partially overcome by feeding magnesium. The altered oxidative phosphorylation of heart muscle was also restored to normal.

It has recently been found that steroid metabolism is intimately related to thyroid function. Patients with thyrotoxicosis dispose of administered steroids such as cortisone more rapidly than normal, and the same with myxedema less rapidly [94]. This has been traced to a control of capacity of the liver to reduce ring A of the steroid by  $T_4$  [95]. Not only does treatment of rats with  $T_4$  increase the availability in liver homogenate of IPNH for ring reduction but with prolonged treatment there is an increase in the microsomal reducing enzyme as well [96]. Similar results have been obtained for testosterone, hydrocortisone, and others.

### *Effects on Oxidative Phosphorylation*

Most of the chemical energy used by the body is channeled through high energy phosphate bonds especially ATP. Oxygen is consumed in the production of this stored and readily available form of energy. The ratio of bonds formed (phosphate uptake) to oxygen used (P/O ratio) is thus a measure of the efficiency of oxygen utilization.

A significant advance in the understanding of thyroid hormones in relation to cellular physiology was the finding that there is a fall in the P/O ratio (uncoupling) of mitochondrial preparations in vitro when  $T_4$  or a number of analogues is added or when the I/O ratio is measured in tissue removed from animals pretreated with  $T_4$ . Many other chemical substances such as dinitrophenol also uncouple as do optical isomers of L-thyroxine and many analogues and related substances [97-100].

The uncoupling explanation of  $T_4$  action has been an attractive one. The principal arguments advanced against its acceptance are that (1)

many substances uncouple oxidative phosphorylation which are in no sense effective in the treatment of hypothyroidism (2) uncoupling fails to explain the normal state of thyroid function or the beneficial effect of  $T_4$ , (3) the concentrations of thyromimetic substances necessary for uncoupling are somewhat higher than those existing in vivo and (4) whereas  $T_4$  may uncouple this takes place largely at the expense of phosphate bond formation but with little if any increase in oxygen consumption. These objections have been largely answered by Lardy and Maley [97] who have proposed a mechanism for the central and most difficult problem namely, that of the specific beneficial effect of  $T_4$ . They propose that  $T_4$  and analogues act at a specific biochemical locus when in low concentration to uncouple a rate-limiting step in the chain of energy yielding reactions. In this way subsequent steps proceed more rapidly and without over all significant loss in efficiency. Larger concentrations of  $T_4$  permit the uncoupling effect to spread to other reactions and lower the over all efficiency. It cannot be claimed that the objections have been entirely met until further evidence has accumulated but the mechanism of action proposed by Martius Lipmann and Lardy and their colleagues comes closer than any other to accounting for the facts.

### CONTROL OF THYROID FUNCTION

The activity of the thyroid gland is largely under the control of the basophilic cells of the anterior lobe of the hypophysis which secrete thyrotropic hormone (TSH). Removal of the hypophysis reduces but does not entirely eliminate thyroid function [101]. TSH has not yet been obtained in pure form and its mode of action on the thyroid is not entirely clear. All measured functions of the gland are stimulated by TSH including both uptake of iodide and secretion of hormone [102].

The rate at which TSH is secreted depends upon the concentration of thyroid hormones in the circulating blood. An increase in plasma concentration of  $T_4$  causes a decrease in secretion of TSH and vice versa in a way provocatively analogous to feed back control of synthesis of enzymes in certain bacterial systems [103]. Substances other than  $T_4$  which have none of the beneficial effects of  $T_4$  but which uncouple oxidative phosphorylation may also inhibit the secretion of TSH [104].

Thyrotropic secretion can also be influenced by the hypothalamus [105-107]. The contribution of the hypothalamic center if any to normal thyroid function or to thyroid activity in disease states of the thyroid is by no means clear. There is no question but that cold and other physiologic stimuli of thyroid function employ the hypothalamic pathway to produce their effects on the gland [108].

The concentration of  $T_4$  in the plasma may exert a measure of control directly on the thyroid [109]. Thyroid function and growth are influenced by the amount of  $T_4$  given to hypophysectomized rats maintained on

constant doses of TSH. The possibility that disposal of the TSH was influenced by the  $T_4$  was not excluded in these experiments.

It has already been pointed out that the rate of hormone synthesis in the thyroid is controlled in part by the amount of stored iodine within the gland [16-17]. There is no satisfactory explanation for this mechanism of control, nor is it well understood how it is that large amounts of iodide inhibit secretion of thyroid hormones by the hyperfunctioning thyroid of Graves' disease.

## TYPES OF FAMILIAL GOITER

The preceding paragraphs have made it evident that there are many steps in the synthesis, storage, and secretion of the thyroid hormones. Many, if not all, of these steps depend upon the functional integrity of specific enzymatic systems. Conceivably, thyroid disease could arise from defective function of any one of these steps or should a step be blocked or ineffective, production of thyroid hormones would be diminished or absent. Compensatory growth and increased activity of the gland might or might not restore adequate formation of hormones.

Several types of familial goiter are now recognized which can be classified with more or less certainty according to specific failure of a normal intracellular process. The patients may be grouped as follows: (1) those whose thyroids are unable to convert inorganic iodide into an organic form; (2) a closely related group of patients who have a similar metabolic fault but who differ from the first group in severity and in having familial eighth nerve deafness; (3) those who are unable to couple MIT and DIT into  $T_3$  and  $T_4$ ; (4) those who have a general tissue lack of iodotyrosine deiodinase; (5) a group which may be heterogeneous and which is characterized by secretion of an abnormal iodinated peptide into the serum. There are many reports of patients who doubtless belong in one or the other of these categories but who cannot be classified because of the meager data available. Still other categories doubtless remain to be defined.

### FAMILIAL GOITER FROM FAILURE TO FORM ORGANIC IODINE

#### *Case Studies*

Five cretins with goiter who share a common defect in organification of iodine have been studied in the author's laboratory [110-112]. Four of these were siblings from a family of seven children. Three older siblings were normal and without thyroid disease and now have a number of normal children. The parents were half Caucasian and half American Indian and were first cousins on the Indian side of the family. Both pairs of paternal grandparents were also first cousins. Growth and de-

velopment of the affected children were reported normal during the first few months of life but thereafter became retarded. Thyroid medication was begun in all between the ages of a few months and 2 years but was intermittent because of the impoverished state of the family. A mass was observed in the neck of each between the ages of 7 and 13 and slowly increased in size until they were seen in the hospital.

*Patient 1* was 16 years old in 1949 at the time of initial studies. She was dwarfed and grossly retarded mentally. The thyroid was enlarged five or six times normal size; it was nodular and firm. A bruit and thrill were present and the skin over the thyroid felt warm to touch. The protein bound iodine (PBI) concentration of the blood was  $1.02 \mu\text{g}$  per 100 ml.

When the patient had been without thyroid medication for 3 weeks, 100 microcuries of  $^{131}\text{I}$  was given orally. The labeled iodine in the gland reached a plateau in less than 2 hr. Twenty-seven hours later, 1.0 gm of KSCN was given by mouth. This caused an immediate and striking fall in the labeled iodine in the gland (Fig. 9-2). At the end of 2 hr the counts had fallen to 75 per cent of the previous level.

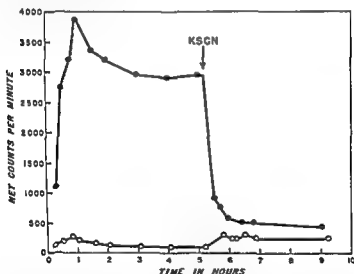


Fig. 9-2. Discharge of iodide from the thyroid gland of a patient with cretinism and hyperthyroidism after administration of thiocyanate.  $^{131}\text{I}$  was given at zero time. Thiocyanate was given orally as indicated by the vertical arrow.

At thyroidectomy the gland weighed 97 gm. There was intense hyperplasia coupled with cystic degeneration and fibrosis (Fig. 9-3). An analysis of the tissue by Douglas Riggs disclosed a concentration of thyroid inorganic iodide of  $1.093 \mu\text{g}$  per 100 gm and of protein bound thyroid iodine of  $10.4 \mu\text{g}$  per 100 gm. The normal thyroid gland contains from 4 to 15 mg iodine of which 1 to 2 per cent is inorganic.

One of the normal siblings of this patient was similarly studied. Following a tracer of  $^{131}\text{I}$  labeled iodine slowly accumulated in the thyroid and reached



constant doses of TSH. The possibility that disposal of the TSH was influenced by the  $T_4$  was not excluded in these experiments.

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Two hundred microcuries  $I^{131}$  was given intravenously. Maximum accumulation was observed in the thyroid almost immediately. The counting rate extrapolated to zero time corresponded to 68 per cent of the administered dose. The quantity of the labeled iodine in the gland declined at a rate estimated at 2.5 per cent per hr ( $T_{1/2} = 27.6$  hr) for the first few days of observation and then appeared to approach an exponential fall at a rate of 1.9 per cent per hr ( $T_{1/2} = 35.9$  hr).



Fig. 9-4. A 3-year-old cretinous male with goiter (Patient 2 of text) (By permission of *The Journal of Clinical Endocrinology and Metabolism*).

The labeled iodine of the blood was measured frequently for the first few hours after administration and daily thereafter. After a few hours the disappearance of labeled iodine from the serum followed a simple exponential having a rate constant of 1.9 per cent per hr. The extrapolated concentration of iodide in the serum at zero time was 2.5 per cent per liter. The volume of distribution was therefore 40 liters (100 per cent  $\div$  2.5). Since 68 per cent of the administered dose was present in the gland at zero time, it is evident that the inorganic iodide compartment of the thyroid accounted for 27.2 liters and that the extrathyroidal iodide compartment was 12.8 liters or 0.31 liters per kg body weight. The last two values are within normal limits.

a maximum value in approximately 21 hr. Oral administration of 2 gm KSCN caused no discharge of labeled iodine from the gland.

*Patient 2*, a 23-year-old cretinous male sibling of Patient 1, was brought to the hospital because of a huge goiter and increasing difficulty in breathing (Fig. 9-4). A PBI determination of the serum was zero.

The patient was given a tracer dose of 100 microcuries of  $I^{131}$  5 months after he had last taken desiccated thyroid. Four and one-half hours later

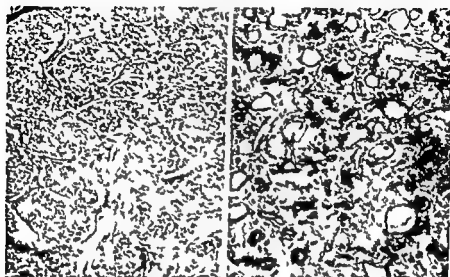


Fig. 9-3 Histologic pattern from two areas of a thyroid gland removed from a patient with congenital goiter. Note the hyperplasia.

when the counts over the thyroid had reached a plateau, he was given 2 gm KSCN by mouth. There was an immediate rapid fall in the recorded counts over the gland and a rise in the radioactivity recorded over the lower back. After 2 hr the counts in the neck had fallen by 70 per cent.

The removed thyroid weighed 497 gm. Pathologic examination showed findings similar to those in Patient 1 above. In many areas there was a paucity of acinus formation characteristic of fetal adenoma, and in others disorganization of cellular pattern and fibrosis. The total inorganic iodine was  $173 \mu\text{g}$  per 100 gm, and the thyroid PBI was  $15 \mu\text{g}$  per 100 gm.

*Patient 3*, a 13-year-old goitrous male sibling of the previous two patients, was brought to the hospital for study. His intellectual attainments were somewhat beyond those of his cretinous siblings. He had learned to feed himself and to walk but had never gone to school.

Labeled iodine accumulated rapidly in the thyroid during the first hour after administration and then slowly declined. Two grams of KSCN orally caused an immediate discharge of the labeled iodine from the thyroid. By 1 hr the counts were approximately 10 per cent of the previous level. The concentration of PBI in the serum was  $1.4 \mu\text{g}$  per 100 ml. The excretion of iodine in the urine varied from 86 to  $232 \mu\text{g}$  for 24 hr (normal).

This patient was studied again 4 years later. The PBI concentration of the serum was  $0.9 \mu\text{g}$  per 100 ml. The thyroid gland was fully five times normal size and was multimodular.

*Patient 6* a 13 year-old female was unrelated to the previous patients. A diagnosis of cretinism was made at the age of 5 months and thyroid medication was given until several weeks before the studies described below were made. Enlargement of the thyroid gland was noticed 1 year prior to admission. Serum PBI concentration was 0.4 and 0.4  $\mu\text{g}$  per 100 ml on successive analyses.

Radioactive iodine accumulated rapidly in the region of the thyroid gland and reached a maximal value of 1 hr. Two grams of KSCN was given by mouth. Shortly thereafter there was a rapid disappearance of the labeled iodine from the region of the thyroid gland. Subsequently the thyroid was removed. The gland was composed of adenomas of varying degrees of cellular differentiation. Some were solid masses of large thyroid cells in packed clusters and cords. Others showed evidence of acinus formation with colloid production. The degree of hyperplasia in many areas was intense and exaggerated. The excised gland contained 0.62  $\mu\text{g}$  total iodine per gram of wet tissue and 0.07  $\mu\text{g}$  precipitable iodine per gram of wet tissue.

**Similar Cases Reported by Others** There have been sporadic reports of other patients who appear to have the same pattern of iodide metabolism as that described above. Schultz, Flink, Kennedy, and Zieve [113] reported a 34 year old male cretin with a huge lobular thyroid gland. Three cretinous siblings were not available for study. Protein bound iodine concentration in the serum was 1.4  $\mu\text{g}$  per 100 ml.  $I^{131}$  uptake reached maximum values at  $1\frac{1}{2}$  hr. When 2 gm KSCN was given orally there was a precipitous fall in the labeled iodine of the thyroid and a rise in the  $I^{131}$  concentration of the serum.

Clayton Smith and Lerner [114] studied a family of six Negro children, four of whom were goitrous. The parents were not consanguineous nor did they have thyroid disease. A maternal great aunt and uncle had goiter. The goitrous members of this family differed from those already described in that they were euthyroid by clinical assessment and by laboratory study. One had a PBI concentration above normal but a normal butanol-extractable iodine (BEI). Another of the goitrous siblings had an abnormally wide difference between the BEI and the PBI. The four goitrous siblings all demonstrated a release of labeled iodine when given KSCN. Studies on one of the normal siblings failed to show any effect of KSCN in causing release of labeled iodine, but further uptake was inhibited. Two goitrous siblings with small goiters showed definite but slight discharge of labeled iodine following administration of KSCN. By chromatographic analysis  $T_4$  and iodide were found in the peripheral serum and  $T_3$  as well. No MIT or DIT was found. Histologic study of a biopsy specimen from one of these patients disclosed a variegated appearance. Some areas showed intense hyperplasia whereas others showed colloid involution.

LeLong, et al. [5] reported a 10 year-old retarded male with five normal siblings. There was rapid accumulation of labeled iodine in the thyroid. Two successive doses of KSCN of 1 gm each caused precipitous

Urine was collected in half hour fractions during the first few hours and thereafter in daily quantities. After the first few hours the daily excretion of labeled iodide fell along a simple exponential line closely paralleling those of the blood and urine. The proportion of gland content excreted in the urine per day was 5.15 per cent and was constant throughout.

The hypothesis that all the labeled iodine in the thyroid was in inorganic form was tested in several other ways in addition to the KSCN method. If one assumed that the labeled iodine in the gland was in free exchange with the iodide of the blood then from the volume of distribution of the iodide and from the renal clearance of iodide it would be possible to predict the *in vivo* disappearance of labeled iodine from the gland. Renal clearance was measured on two separate occasions with six half hour collection periods. The mean value was 1.31 liters of plasma per hour. Since the volume of the iodide compartment was 400 liters then 3.29 per cent of the iodide compartment was cleared per hour corresponding to a half time of disappearance of 21.1 hr. The observed *in vivo* disappearance rate was estimated to have a half time of 27.0 hr during the initial few days of the experiment.

If it is assumed that the specific activity of iodine in the gland is the same as that in the blood and urine (an assumption which follows the hypothesis of the preceding paragraph) then from measurements of stable and labeled iodine in the urine it is possible to calculate the quantity of iodine present in the thyroid gland thus

$$Q = \frac{Q_0 \cdot F}{F}$$

where  $Q_0$  = the percentage of the administered dose of labeled iodine in the gland

$E$  = the 24 hr excretion of iodide

$E^*$  = the 24 hr excretion of labeled iodide

The mean value of  $Q_0$  calculated for 10 days was the astonishingly small quantity of 2.7  $\mu$ g.

The total iodine content of the gland was measured after surgery by chemical analysis of four fragments of a half gram each from four separate areas. Over 99 per cent of the iodine was trichloroacetic acid-soluble. The calculated iodine content proved to be 319  $\mu$ g a figure which agrees well with the calculated estimate of iodine content of the whole gland based on the hypothesis that all the iodine contained therein was in inorganic form. Chromatographic analysis of the four fragments of tissue also failed to demonstrate any organically bound labeled iodine.

*Patient 4* the oldest cretinous sibling of the previous patients was first admitted to the hospital at age 13 for a mass in his neck. Two hundred and thirty five grams of thyroid tissue was removed. The histologic appearance was similar to that described for the three siblings. The patient was readmitted to the hospital at age 27 because of regrowth of the goiter and pressure symptoms causing respiratory difficulty. An emergency tracheotomy was required and this was followed by a total thyroidectomy. Approximately 175 gm thyroid tissue was removed. In addition to the same histologic pattern which was evident at the time of the earlier thyroidectomy there was an area of vascular and capsular invasion which was considered to be diagnostic of malignant change. There has been no evidence of recurrent disease in the 11 years of follow up. No other laboratory investigations have been made on this patient.

None was observed although normal human thyroid tissue under identical conditions readily formed MIT. The authors preferred the possibility that the gland had an iodinase defect rather than defective formation of active iodide or that there might be an abnormal receptor protein.

### *Genetics*

Too few families with this disorder have been reported at the present time for a firm interpretation of the genetics of this disease. If one excluded the cases of Clayton et al. on the grounds that their patients were not cretinous, then 10 affected patients have been reported in 4 families of 28 children. The sex of 3 of the affected persons is not given but 2 are female and 5 are male. Although in the patients reported to date there is a slight excess of affected sibling above the expected ratio, the data are not inconsistent with inheritance as a simple autosomal recessive gene. This interpretation is also consistent with the consanguinity in the family of Patients 1 to 4.

The genetic interpretation of Clayton's patients who were not hypothyroid is difficult. It seems unlikely that they could be heterozygous for the same gene for which other members of this group are homozygous because parents of other sibships have not shown evidence of thyroid disease and yet must have been heterozygotes. As already mentioned, it is possible that oxidation of iodide to iodine and fixation to tyrosyl residues form a complex rather than a single-step process and that the response to thiocyanate might be similar in patients having different defects in hormone synthesis.

## FAMILIAL GOITER AND DEAF MUTISM

### *Case Studies*

Since Brain reported 6 families of congenital goiter and deafness in 1927 [118], at least 12 other families have been reported with this association of diseases. With few exceptions the clinical pattern has been uniform. Goiter has been apparent either at birth or within the first decade of life and nerve deafness has been present from birth or has developed during childhood. Intelligence and usually growth have been normal. Vestibular function has been normal or nearly so and the tympanic membranes have been intact.

These patients have not had huge goiters. Thyroid function as assessed by the usual clinical and laboratory data has been normal in most of them. The serum concentrations of PBI in most of the affected persons of Johnson's three kindred were distinctly low [119]. Two of the four affected members of the sibship of Ilman [120] had developed invasive adenocarcinoma and Roberts' case [121] had localized histologic change

but partial falls of the labeled iodine in the gland from the maximal uptake of 57 per cent. Three similar patients have been reported by Cardner et al [115].

A 13 year-old cretin briefly mentioned by Federman et al [116] had a PBI of 0.7  $\mu\text{g}$  per 100 ml and a bone age of 1 year. Maximal uptake of  $\text{I}^{131}$  was 30 per cent at 4 hr and 90 per cent of this was discharged from the gland upon administration of  $\text{KSCN}$ . There was a concurrent rise of labeled iodine in the blood as recorded over the thigh. The cretinous infant described by Jackson [117] may also belong to this group but thiocyanate caused a release from the gland of only approximately 30 per cent. A control study without  $\text{KSCN}$  showed almost complete loss of labeled iodine from the gland in 48 hr.

### *Physiologic Interpretation*

The common denominator of the patients in this group is the release of labeled iodine from the thyroid gland upon the administration of  $\text{KSCN}$ . The clinical findings have not been uniform. The majority have been severe cretins with profound retardation of structural and intellectual development. Those reported by Clayton et al [114] were clinically euthyroid as confirmed by laboratory examination.

The release of labeled iodine from the thyroid by thiocyanate indicates a store of inorganic iodide in the gland which can be readily displaced. Normally, virtually none of the accumulated iodine can be displaced in this way. Those patients who showed a thiocyanate release of labeled iodine appear to have a metabolic defect which makes them unable to convert accumulated iodide to organically bound iodine. Since this limits or prevents hormone synthesis, compensatory hypertrophy and hyperplasia of the thyroid occur, as do the consequences of an insufficient hormonal supply. Support of this interpretation has come from chromatographic and analytic studies. The kinetics of iodine metabolism reported from Patient 3 above were consistent with a large iodide space in the thyroid in free communication with the iodide space in the blood and inconsistent with any store of bound iodine in the thyroid gland. Tissue analyses on three of the patients in that kindred indicated virtually no organically bound iodine in the gland.

The biochemical lesion in the thyroid of these patients seems established inferentially but not precisely or directly. Unquestionably there is interference or failure in the step where iodide becomes oxidized to iodine and displaces a proton from a tyrosyl residue. The enzyme or enzymes which may be involved have been discussed earlier. The evidence is in accord with an absence of the specific oxidative enzymatic process in the thyroid gland which is responsible for conversion of iodide ion to  $\text{I}_2$  (or  $\text{HIO}$ ). Haddad and Sidbury [114a] have had the opportunity to measure the capacity of a gland from a similar patient to form MIT.

[120a] Similar findings were obtained in one similar patient by Buchanan and Crooks [123a]. In their second patient there was no discharge after administration of perchlorate but in spite of a 75 per cent uptake of  $I^{131}$  by the thyroid no PBI<sup>131</sup> was found and the labeled iodine was largely gone from the gland at the end of 24 hr.

Among the reported patients in this group no members of the families have goiter without deafness or the reverse. The sex distribution is nearly equal. Morgans and Trotter pointed out that this is unusual since ordinarily thyroid disease is much more common in females than in males. Only in Johnson's kindreds has the disease appeared in successive generations (Fig. 9-5).

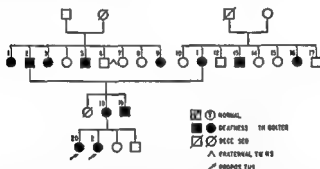


Fig. 9-5. Inheritance of deafness and goiter in an intermarried family studied by Johnson [119] (Redrawn and printed by permission from *Acta oto-laryngologica*).

The inheritance of this disease is not entirely clear from the family charts available. Approximately one-half the sibs in the reported families have been afflicted. Although the pattern is not entirely inconsistent with an autosomal dominant form of inheritance it is probably best at the moment to withhold judgment. It is by no means clear why the thyroid and the eighth nerve are both involved. Close linkage of the relevant genes could obtain or both goiter and deafness could be the result of pleiotropic action of a single gene.

#### CRETINISM WITH FAILURE OF CONDENSATION OF IODOTYROSINES

##### Case Study

Defective coupling of MIT and DIT into  $T_3$  and  $T_4$  was the cause of hypothyroidism and goiter in a 23-year-old retarded female studied in the author's laboratory [11]. A diagnosis of hypothyroidism was made at age 4. Desiccated thyroid was prescribed but taken only intermittently. A goiter was first observed at age 17. At 22 the serum concentration of PBI was  $3.5 \mu\text{g}$  per 100 ml. The daily excretion of  $I^{131}$  was normal at a time when the patient had ingested no desiccated thyroid for the previous 8 weeks. Measurements of the basal metabolic rate and PBI were normal at the time of



suggestive of malignant degeneration. Two of the four patients in Thiem's sibship also had local malignant degeneration without evidence of distant extension [123].

### *Thyroid Function Studies*

Laboratory observations designed to clarify the thyroid function of these patients have been performed principally by Morgans and Trotter [123], by Elman [120] and by McGirr and Hutchison [120a]. Morgans and Trotter gave radioactive iodide to two patients and made serial measurements of labeled iodine over the neck and thigh. An identical study was done on the normal mother of these two siblings. One hour after administration of radioiodine each of the two patients received 200 mg of  $\text{KClO}_4$  in water by mouth. Within the next 30 min there was a fall of approximately 30 per cent in the counting rates recorded over the neck and a rise over the thigh. The administration of perchlorate to the mother of the two patients was attended by little change either in neck or thigh counting rates. It was concluded from these observations that these patients had a partial defect in the organization of labeled iodine. Similar results and interpretations derive from the studies of McGirr and Hutchison [120a].

The studies of Elman were more limited in scope. He observed that in one of his patients the  $\text{I}^{131}$  uptake in the neck was 30 per cent in 1 hr and 23 per cent in 24 hr. In the other patient the respective figures were 20 and 16 per cent. He drew no conclusions from these observations other than to suggest that the fall in labeled iodine of the thyroid between the first and twenty-fourth hours indicated some sort of enzymatic abnormality in the thyroid gland. Further and more detailed studies of similar patients should be rewarding.

### *Genetics*

Morgans and Trotter [123] summarized the literature until 1958 and found 20 affected siblings among sibships totaling 41. Johnson's 2 families included 24 members of whom 21 were personally examined [119] (Fig. 9.5). Of these 12 exhibited goiter and deafness. Four of his cases were the result of intermarriage of 2 affected members of the 2 families. Four of 7 siblings of Elman's sibship were affected. Deraemaeker [124] in Belgium found 3 affected males in a sibship of 12 from a first cousin marriage. Interestingly 2 of these had associated ichthyosis. The author excluded a chance association of recessive genes (or genetic defect) on statistic grounds. Perhaps with less justification he excluded linkage and concluded that the disease was to be understood as an instance of a pleiotropic gene effect of a recessive gene. Discharge of approximately 50 per cent of accumulated  $\text{I}^{131}$  was caused by oral thiocyanate or perchlorate to all five of the recently reported patients of McGirr et al.

Chromatographic analysis of serial serum samples taken between the seventh and seventy ninth hours after administration of  $I^{131}$  disclosed  $T_4$  and  $T_3$  in all samples. Yet in spite of heavy labeling of the gland and rapid turnover of iodine by the gland it was impossible to demonstrate either  $T_4$  or  $T_3$  in four tissue specimens obtained at operation although iodide and abundant MIT and DIT were demonstrated with ease. The specimens were obtained 79 hr after the administration of  $I^{131}$ . One hundred and thirty grams of tissue were homogenized, hydrolyzed, and analyzed for  $T_4$  by the method of Gross and Pitt Rivers [195]. Pitt-Rivers was unable to demonstrate more than a trace of  $T_4$ .

### *Physiologic Interpretation*

Two lines of investigation indicated that the primary fault in the thyroid gland of this patient was an inability to couple MIT and DIT into  $T_4$  and  $T_3$ . The more significant evidence derived from chemical studies on the blood and on the excised thyroid gland. Serial chromatographic analyses of the serum for many hours after the administration of  $I^{131}$  demonstrated the early appearance of  $T_4$  and of  $T_3$  but of no unidentified iodinated substance. Detailed analysis of the glandular tissue failed to disclose labeled  $T_4$  or  $T_3$ . Only when the whole gland was subjected to chemical analysis was it possible to identify a trace of  $T_4$ . Yet at the same time abundant quantities of MIT and DIT were found. In the normal thyroid gland at least 20 per cent if not more of the labeled iodine is in the form of labeled  $T_4$  by 49 hr. Thus it appeared that while this hyperplastic gland was making ample MIT and DIT and was storing them in large amounts in the gland, the  $T_4$  and  $T_3$  which were produced were immediately released into the blood. There was evidence therefore of an abundant formation of hormone precursors in the form of iodotyrosines but of limited formation of iodothyronines.

Supporting information was obtained from kinetic studies of iodine metabolism in this patient. For the details of the observations and their interpretation the reader may consult the original publication [112]. It was possible to measure the rate at which iodine was leaving this gland in two ways. During administration of methimazole the specific activity of the labeled iodine which appeared in the urine was extremely high. This proved to be 14 per cent per mg at a time when the specific activity of the whole gland was only 2.9 per cent per mg. This finding could only be interpreted as indicating that methimazole was blocking the reutilization of labeled iodine derived from a pool of high specific activity whereas most of the labeled iodine in the thyroid was in a pool of low specific activity.

The amount of unlabeled iodine leaving the gland daily and coming into equilibrium with the extrathyroidal iodine compartments was estimated from the equilibrium expression

$$H = \frac{EI}{1 - I}$$

where  $H$  = the quantity leaving the gland

$E$  = the daily excretion of  $I^{131}$

$I$  = the fractional uptake

Although  $H$  could not be estimated precisely because of the sensitivity of the excretion to small changes in  $I$  when  $I$  is close to 1 as it was in this patient, still the calculated value of  $H$  was at least 1 mg. The normal

study. A total thyroidectomy was performed at the completion of studies. Microscopically the specimen displayed extreme hyperplasia.

The family history was of particular interest because it displayed two first-cousin marriages (Fig 9-6) in the immediate antecedents of the patient and because a sister 4 years older suffered from an identical disorder. Daily excretion of  $I^{127}$  was normal on several occasions. Concentration of IBI in the serum was  $6.1 \mu\text{g}$  per 100 ml. She had undergone an uneventful subtotal thyroidectomy because of rapid growth of the gland. Pathologic examination disclosed multinodular goiter with extreme hyperplasia and almost no colloid formation within the follicles. Earlier studies on both sisters had shown an unusually rapid uptake of  $I^{127}$  to values in excess of 95 per cent. The labeled iodine in the thyroid was not dislodged from either patient by administration of  $\text{KSCN}$ .

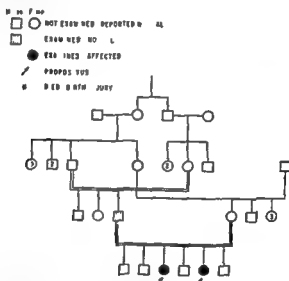


Fig 9-6 Inheritance pattern of a family with two siblings with congenital goiter (see text). Note the two consanguineous marriages.

The special studies performed included detailed examination of the metabolism of iodine by measurements of the uptake, retention and release of labeled iodine and the excretion of stable iodine and by chemical examination of the histologic specimen at the time of surgery. Labeled iodine accumulated rapidly in the thyroid to a maximum of 99.3 per cent within the first 2 hr. Thereafter it was released at a rate approaching 3.0 per cent per day. When methimazole was given there was a sharp increase to 7.3 per cent per day. When methimazole was withdrawn the release rate slowed. The iodide clearance by the kidney was 30.5 ml per min. The renal excretion rate of labeled iodine rose sharply upon the administration of methimazole; also the daily excretion of stable iodine rose from normal values to a mean daily  $I^{127}$  excretion of  $203 \mu\text{g}$  per day, above mean control values. The labeled iodine in the plasma fell sharply immediately after administration of  $I^{127}$  but began to rise after a few hours and reached a maximum on the fifth day of  $0.52$  per cent per liter and then slowly fell.

Chromatographic analysis of serial serum samples taken between the seventh and seventy ninth hours after administration of  $I^{131}$  disclosed  $T_4$  and  $T_3$  in all samples. Yet in spite of heavy labeling of the gland and rapid turnover of iodine by the gland it was impossible to demonstrate either  $T_4$  or  $T_3$  in four tissue specimens obtained at operation although iodide and abundant MIT and DIT were demonstrated with ease. The specimens were obtained 79 hr after the administration of  $I^{131}$ . One hundred and thirty grams of tissue were homogenized, hydrolyzed, and analyzed for  $T_4$  by the method of Gross and Pitt Rivers [1-5]. Pitt-Rivers was unable to demonstrate more than a trace of  $T_4$ .

### *Physiologic Interpretation*

Two lines of investigation indicated that the primary fault in the thyroid gland of this patient was an inability to couple MIT and DIT into  $T_3$  and  $T_4$ . The more significant evidence derived from chemical studies on the blood and on the excised thyroid gland. Serial chromatographic analyses of the serum for many hours after the administration of  $I^{131}$  demonstrated the early appearance of  $T_4$  and of  $T_3$  but of no unidentified iodinated substance. Detailed analysis of the glandular tissue failed to disclose labeled  $T_4$  or  $T_3$ . Only when the whole gland was subjected to chemical analysis was it possible to identify a trace of  $T_4$ . Yet at the same time abundant quantities of MIT and DIT were found. In the normal thyroid gland at least 90 per cent if not more of the labeled iodine is in the form of labeled  $T_4$  by 79 hr. Thus it appeared that while this hyperplastic gland was making ample MIT and DIT and was storing them in large amounts in the gland the  $T_4$  and  $T_3$  which were produced were immediately released into the blood. There was evidence therefore of an abundant formation of hormone precursors in the form of iodotyrosines but of limited formation of iodothyronines.

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The amount of unlabeled iodine leaving the gland daily and coming into equilibrium with the extrathyroidal iodine compartments was estimated from the equilibrium expression

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where  $H$  = the quantity leaving the gland

$E$  = the daily excretion of  $I^{131}$

$l$  = the fractional uptake

Although  $H$  could not be estimated precisely because of the sensitivity of the expression to small changes in  $l$  when  $l$  is close to 1 as it was in this patient still the calculated value of  $H$  was at least 1 mg. The normal

amount of hormonal iodine secreted per day lies between 50 and 150  $\mu$ g. Only a small fraction of the iodine leaving the gland could have been in the form of thyroid hormone because otherwise the patient would have been thyrotoxic. Accordingly, the thyroid must have been releasing iodide or a readily metabolized iodinated substance which was quickly broken down either by the gland itself or in the periphery to yield iodide. No other interpretation seems to fit the data.

The simplest formulation to account for the facts was that the coupling of DIT to yield  $T_4$  took place only to a limited degree, perhaps because of partial or total deficiency of a coupling enzyme. The  $T_4$  which was formed was secreted rapidly, perhaps without being staged through thyroglobulin. In this way it is possible to account for the almost complete absence of  $T_4$  from the gland despite normal concentrations in the peripheral blood. The highly stimulated gland maintained an exceedingly rapid flux of some of its iodine. Some went on to form hormone, some was retained for storage as MIT and DIT, and a large fraction—perhaps in the form of MIT—was formed and released for deiodinization either in the gland or in the periphery or both.

It has not been possible to demonstrate a defect in  $T_4$  coupling directly because the precise biochemical pathway by which this takes place has not been defined. That the disease occurred in two sisters studied by the author strongly suggests a common biochemical defect under genetic control.

### *Similar Cases Reported by Others*

Two unrelated female patients were carefully studied by Mosier, Blizard, and Wilkins [196]. One was 13 and the other 37. Both were hypothyroid and retarded and both had large goiters. Serum concentration of protein bound iodine were low. Thyroidal uptake of  $I^{131}$  was rapid in both and peak values were reached within 3 hr. The uptake was normal in the younger patient and low in the older. Administration of KSCN caused little change in the labeled iodine in the gland. No abnormal iodinated substances appeared in serum or urine after administration of  $I^{131}$ . There appeared to be diminished deiodination of intravenously administered labeled DIT when the patients were hypothyroid, but this was restored to normal when they were receiving full doses of desiccated thyroid. Homogenates of the surgically removed glands deiodinated DIT normally.

The significant findings were in the glands. Chromatographic analysis of the tissue disclosed that the labeled iodine was in the form of MIT and DIT. Almost none was present as labeled  $T_4$ , although  $T_4$  had been demonstrated in the peripheral blood. Although only 24 and 30 hr elapsed between administration of  $I^{131}$  and surgical removal of the two glands, by this time much more of the labeled iodine would normally have been in the form of  $T_4$  than was in fact found. Mosier et al. concluded that the metabolic defect in these two patients was a block in the coupling of iodotyrosines into  $T_4$  and  $T_3$ .

Similar patients have been described by Joseph and Job [127]. Two females from a sibship of 10 were hypothyroid and had goiter. Each patient had a high uptake of  $I^{131}$  and prolonged retention in the gland. There was no discharge from the gland upon the administration of SCN<sup>-</sup>. When  $I^{131}$  labeled DIT was administered intravenously, the principal labeled iodine which appeared in the urine was inorganic in form. Chromatographic analysis of a biopsy specimen from the thyroid of one of these patients disclosed

that 60 to 70 per cent of the labeled iodine was in the form of DIT and 21 to 25 per cent in the form of MIT. The rest of the iodine was present as iodide and if  $T_4$  or other iodothyronines were present they were in a concentration of less than 1 per cent of the total.

A puzzling case which may belong in this category has been reported by Werner et al [123]. Their patient was a 5 year old female without retarded development. Uptake of  $I^{131}$  by the large goiter was repeatedly found to be elevated and turnover by the gland was rapid. The serum concentration of PBI was 28.4  $\mu$ g per 100 ml. Labeled MIT, DIT,  $T_3$  and  $T_4$  were identified in the serum by the chromatographic methods employed, but degradation of intravenously administered labeled DIT was normal and no abnormal iodinated substances were found in the urine. Analysis of the gland removed 24 hr after administration of  $I^{131}$  disclosed MIT and DIT but only small quantities of iodothyronines. Werner et al concluded that their patient suffered from a defect in MIT and DIT coupling with subsequent spillage of these into the blood. An 8½ year-old female with certain similarities has been reported in abstract by Whitelaw et al [120]. Studies of other patients in this category have shown neither the iodothyronines nor the remarkable elevation in serum concentration of PBI.

The patients of Joseph and Job and of Mosier et al correspond closely in most details with those from the author's laboratory. The author's patient and her sister had achieved a euthyroid state but earlier clinical observations had indicated clearly that they had been hypothyroid. There are numerous reports in the literature of patients who conform to the pattern of these patients clinically and who also have rapid and high uptake levels of radioactive iodine [130-134]. The cretinous brothers studied by Lerman et al [130] were of particular interest because they were the first to be studied physiologically and because of the intense hyperplasia which the glands displayed. They are well and free of goiter at the present writing.

Until simpler and more reliable methods are developed for defining patients of this group at a molecular level it seems unlikely that many can be placed with certainty in this category. At the present time the minimal criterion for inclusion depends upon chromatographic analysis of the labeled iodine in the thyroid removed more than 3 days after administration of the labeled iodine. Labeled iodothyronines are found at most in trace amounts; whereas labeled iodothyronines are present in abundance. A word of caution is in order here: large sluggish colloid filled glands may form  $T_3$  and  $T_4$  very slowly from MIT and DIT.

### Genetics

Little can be said regarding the genetics of this group. The author's 2 patients had no family history of thyroid disease except for their own sibship but there were two first cousin marriages among their antecedents. The family described by Joseph and Job [127] with 11 afflicted

members out of 10 in the sibship suggests a simple recessive gene. All 6 of the patients who can be accepted at present are female.

## CRETINISM FROM FAILURE OF IODOTHYRONINE DEIODINASE ACTIVITY

### Case Studies

In 1953 Hutchison and McGirr [155] reported 12 patients from the Royal Hospital for Sick Children and the Royal Infirmary in Glasgow, Scotland, who were cretinous and hypothyroid. Four of these were siblings and 3 and 1 subsequently described [156] were from the same kindred. These 8 patients were of itinerant tinker stock, an isolated group of people living a nomadic existence in Argyshire and the Isle of Slay. Two others of the cretins were half-siblings and were also of tinker stock but from Perthshire and Angus.

The tinker cretins were all goitrous. The uptake of  $I^{131}$  of all but 2 was high and the maximum value was quickly reached. Most of the glands lost a large fraction of the accumulated labeled iodine within a few hours after maximum uptake. Only 2 failed to show a high concentration of PBI<sup>131</sup> in the peripheral blood at 48 hr. Analyses of the serum of 3 of these patients disclosed an organic butanol-extractable substance. This component was not identified but the possibility was entertained that it might be DIT. Chromatographic studies of the serum of 1 patient failed to identify the component as an iodothyronine.

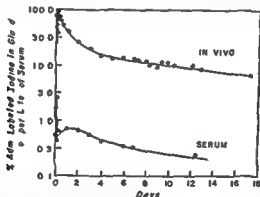


Fig. 9-7 Metabolism of iodine in Patient V-1. I was given orally at zero time and serial counts made over the thyroid and in the serum.

Three similar patients were studied at the Academisch Ziekenhuis in Leiden in 1955 [157-158]. The first of these was a 27-year-old male (Patient A) who was found to have a goiter at birth. Thyroid medication was given for many years but was discontinued 4 years prior to entry to the hospital because of tuberculosis. Since that time the goiter had increased relentlessly in size. The serum concentration of PBI was  $0.5 \mu\text{g}$  per 100 ml. His parents were not consanguineous and were normal, as was an older brother but a younger brother (Patient B) who was also available for study had a goiter at birth and was retarded both physically and mentally. A third unrelated patient (Patient C) was a 12-year-old female cretin with a huge multinodular goiter, short stature, and mental deficiency.

The initial study of Patient A concerned the fate of an administered dose of  $I^{131}$  [137]. Labeled iodine accumulated unusually rapidly in the thyroid (Fig 9.7). Within 70 min 74 per cent had appeared in the neck region and during this time there was a rapid fall in the serum concentration of labeled iodine. After 70 min the labeled iodine began to leave the thyroid and to appear in the blood as a component which was partially precipitable with trichloroacetic acid. By 48 hr only 25 per cent of the administered dose remained in the gland. The serum concentration of labeled iodine had risen

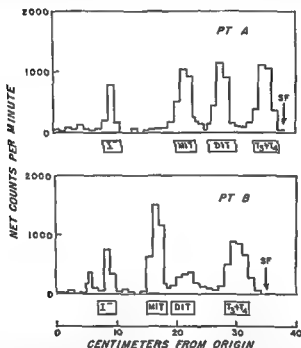


Fig 9-8 The labeled substances appearing in the blood of two brothers with congenital goiter following administration of  $I^{131}$ . The chromatograms were developed in the butanol-acetic acid solvent system. The block symbols represent the position on the chromatogram taken by the added markers. Note particularly the presence of limited amino acids in the serum.

to 0.5 per cent per liter and from that point it slowly fell during succeeding days. Nearly identical *in vivo* retention and release curves were obtained from Patients B and C [138].

Specimens of serum obtained at 2, 4, 8, 16, and 22 hr were extracted with butanol and chromatographed in a butanol-dioxane-ammonia solvent system. In successive specimens there were increasing amounts of labeled iodine which had the chromatographic mobility of MIT and DIT. Moderate amounts of T<sub>3</sub> and T<sub>4</sub> were also found. Confirmation of the presence of MIT and DIT was obtained in a butanol-acetic acid solvent system. The largest fraction of the labeled iodine in the serum was present as these two components (Fig 9.8).



The gland was removed because of pressure symptoms. The histologic pattern was that of intense hyperplasia but some areas showed a tendency to form colloid and there were also areas of cystic degeneration and fibrosis. The gland weighed 198 gm at operation. Three and four tenths per cent of the labeled iodine was recovered as T<sub>4</sub>. The remainder was found in small amounts as iodine and in large amounts as MIT and DIT.

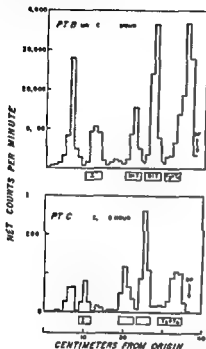


Fig 9-9 The labeled compounds appearing in the urine following oral administration of I<sup>131</sup> to two patients with cretinism and goiter. Note the presence of iodinated amino acids, large quantities of labeled substances moving near the solvent front, and a large zone of labeled iodine near the origin. The last was hydrolyzed with HCl to MIT.

was studied in a group of 15 subjects, and the fate of intravenously administered L-MIT was studied in three subjects without thyroid disease [139]. The results confirmed the earlier studies of Albert and Hering [140], who found that after intravenous administration of I<sup>131</sup> labeled DIT, only a small fraction of the labeled iodine is excreted in the urine as free iodide. Labeled DIT is deiodinated *in vivo*, and labeled iodide increased in the serum relative to labeled DIT. Within an hour after administration the relative amount of labeled iodine of the serum as DIT fell from approximately 90 per cent to approximately 65 per cent. The labeled iodine was excreted in the urine largely as inorganic iodide (Fig 9-10). During the first few hours after administration the largest percentage of the administered dose which ap-

The blood findings on Patient A were duplicated in his brother (Patient B). After administration of I<sup>131</sup>, large amounts of labeled iodine which was not inorganic in form appeared in the serum. Several iodinated components were present on chromatograms of successive urine specimens (Fig 9-9). One of these appeared between the origin and the iodide zone in the butanol-acetic acid solvent system. Another traveled near the solvent front. In addition, labeled iodine was present at the MIT and DIT zones. The component near the origin was readily hydrolyzed into MIT by treatment with dilute acid, but the conjugated substance was not positively identified.

Observations on Patient C were more limited in scope. The *in vivo* thyroidal uptake reached 70 per cent in 1 hr and then fell rapidly. The same iodinated components in approximately the same proportion were found in successive urine specimens as were found in specimens from Patient B (Fig 9-9).

Subsequently, the fate of injected labeled MIT and DIT was observed in Patients A, B, and C, and in several relatives of Patient C. In preparation for these studies the normal metabolism of labeled MIT and DIT

peared in the urine of any patient as DIT was 7.3 and most of the values were below 3. The only patient with a value above 3 per cent was one with diabetes mellitus and glycosuria.

The results of experiments with labeled L-MIT in normal subjects were similar excepting that scarcely any detectable MIT appeared in the urine. A small zone of labeled iodine was found between the iodide zone and the

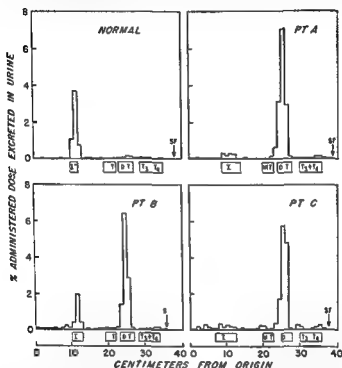


Fig 9-10 Chromatograms of urine after intravenous administration of labeled DIT to a normal person and to Patients A, B, and C. The administered DIT appears as iodide in the normal person and almost entirely as DIT in the three patients.

origin in chromatograms which were developed in the butanol-acetic acid solvent system. When the racemic form of labeled MIT was administered a moderate amount of labeled iodine appeared near the solvent front, and a large fraction was recovered in the urine as MIT. Thus it appeared that both L-MIT and L-DIT were rapidly degraded upon intravenous administration, and that the labeled iodine which appeared in the urine was almost entirely in the form of inorganic iodide.

$^{131}\text{I}$  labeled DIT was given intravenously to Patients A, B, and C. The results were the same in all three (Fig 9-10). Almost all the injected labeled iodine was excreted within the first 24 hr, and almost all of it was excreted as diiodotyrosine. There was no evidence that any of the DIT was deiodinated by any one of these three patients, and there was little evidence of conjugation or deamination. A small and questionably significant amount

of labeled iodine was found near the solvent front. In the first 24 hr after administration 74.6, 78.1 and 66.2 per cent of the injected labeled DIT was recovered unchanged in the urine of Patients A, B and C.

These patients also were given DL-MIT intravenously. In contrast to normal individuals the patients showed practically no excretion of labeled iodide. Almost all the labeled iodine appeared in the urine either as MIT or as zones of labeled iodine near the solvent front or near the origin in the butanol-acetic acid solvent system.

Limited but similar studies were permitted on the sister, mother, two maternal aunts and one maternal cousin of Patient C. The sister, age 20, was entirely normal except for a moderately firm enlargement of her thyroid gland to about twice normal size. The mother of Patient C, age 43, had a subtotal thyroidectomy at 27 for progressive thyroid enlargement but there was recurrent growth of the gland to about five times normal size at the time of study. The patient otherwise was normal and her serum concentration of PBI was normal. One of the maternal aunts had a thyroidectomy for an enlarged thyroid gland which had been diagnosed as Graves disease. The other maternal aunt was said to have had a goiter in the past but this had disappeared. One of her daughters (cousin of Patient C) had a surgical thyroidectomy at age 20 and had a recurrent goiter of about four times normal size at the time of study. A sibling (cousin of Patient C) had died at age 9 on the day prior to a scheduled thyroidectomy for Basedow's disease. In each of the five members of the kindred a dose of labeled DIT was given and the excretion rate measured during the subsequent 4 hr. The results were compared with those obtained from the 15 patients without thyroid disease discussed earlier. It was found that the mean excretion rate of unchanged labeled DIT was significantly higher than in the control group although a large fraction of the DIT was deiodinated (Table 3-1).

TABLE 3-1 EXCRETION RATE OF UNCHANGED LABELED DIT

| Subjects                             | Per cent injected dose DIT excreted as DIT |        |        |
|--------------------------------------|--|--------|--------|
|                                      | 0-1 hr                                     | 0-2 hr | 0-4 hr |
| 15 control patients                  | 1.8  | 4      | 2.8    |
| 3 patients with congenital goiter    | 21.2                                       | 46.2   | 62.3   |
| 3 relatives of one of above patients | 4.6  | 6.2    | 7.1    |

When it became apparent that the thyroid of Patient B would be removed because of pressure symptoms, the capacity of beef thyroid tissue to deiodinate labeled DIT was studied. The method employed was that of Roche et al. [68]. Tissue slices were incubated for 18 hr in the presence of  $I^{131}$  labeled L-DIT. Approximately 60 per cent of the substrate was deiodinated. Similar findings were obtained with slices from human thyroids received at the time of surgical removal because of nodular goiter [141].

The thyroid gland of Patient B then became available. On the day of operation a nodular goiter from another patient was also available from the operating room. Identical experiments were set up to demonstrate the deiodinating capacity of tissue slices from these two glands. A normal amount of deiodination was demonstrated in the nodular goiter but the

thyroid tissue slices from Patient B failed to deiodinate the substrate DIT [141]

Detailed analytical studies of two of the tinker cretins of McGurr and Hutchison [135-136] have clearly shown that they have the same type of thyroid disorder as Patients A, B, and C. Chromatograms of serum samples of one of their patients disclosed MIT and DIT as well as iodothyronines. MIT, DIT, and two unidentified zones of labeled iodine were found in chromatograms from both urine specimens [142-143]. McGurr and Hutchison have pointed out quite rightly that the disorder is the result of a single genetic defect and deserves to be categorized after Garrod as another inborn error of metabolism [136].

Almost simultaneously with the publication of the studies on Patients A, B, and C, Horst [144] published a brief account of his observations on two goitrous cretins. MIT and DIT were identified in serum samples. Horst offered the same interpretation of his patients as for those already described [138].

Costa et al. [145] have chromatographic evidence of DIT in the serum of three cretins from a region of endemic goiter in northern Italy. Patients with endemic goiter do not customarily have MIT or DIT in blood or urine. The *in vivo* curves obtained on two unrelated retarded goitrous subjects by Bernheim and Berger [146] are similar to those on Patients A, B, and C. Burrell and Gardener [147] suggested that their three cretinous siblings (one with goiter) might be secreting DIT. The thyroids of their patients had strong avidity for  $I^{131}$  and the serum concentration of  $PBI^{131}$  was much elevated at 48 hr.

### *Physiologic Interpretations*

Patients of this category share a defect in their capacity to deiodinate MIT and DIT. The deiodinating enzyme has been described normally not only in the thyroid but in liver, kidney, and other organs. The metabolic defect is both intra- and extrathyroidal for not only are the afflicted patients unable to deiodinate MIT and DIT in their thyroid glands but when these substances are administered either before or after thyroidectomy or to the patient receiving full doses of desiccated thyroid, the labeled iodotyrosine appears almost quantitatively in an unchanged form in the urine or in the case of MIT in conjugates.

An objection has been raised that the hypothesis of a single enzymatic defect in these patients is insufficient to account for the poor production of  $T_4$  and for the hypothyroidism [6]. It has been suggested that there must also be an associated defect in the coupling of iodotyrosines into  $T_3$  and  $T_4$ . This seems unnecessary and improbable. In at least one of our patients, significant amounts of labeled  $T_4$  and  $T_3$  were detected in the peripheral blood. The intensely hyperplastic hyperfunctioning glands are thought to secrete hormone precursors as fast as they are formed, leaving little in the gland for coupling into iodothyronines. Theoretically, the provision of unusually large amounts of iodide might enable these thyroids to produce normal amounts of hormone. Observations on this point have not been made.

## Genetics

An unusual opportunity to study the genetics of this kind of goitrous cretinism has been beautifully exploited by McCurr and Hutchison [148]. Exhaustive studies of this closely knit and culturally isolated group in western Scotland have traced the complex family history through 160 years. The original male member came from Ireland and married his full cousin. There has been little marriage subsequently outside the tinkers group, but intermarriage within the group has been extremely frequent.

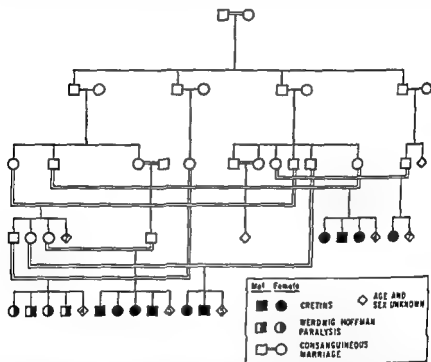


Fig. 9-11 Pattern of heredity of cretinism with goiter as found by McCurr in a group of itinerant Scottish tinkers. Note particularly the remarkable number of consanguineous marriages. (by permission of *The American Journal of Medicine*)

as can be seen in the genetic chart (Fig. 9-11). Ten goitrous cretins are known to have appeared among 111 persons in four sets of siblings. In addition, there have been four cases of Werdnig-Hoffman paralysis.

A study of the family tree shows that this form of cretinism with goiter behaves as a simple autosomal recessive gene. There is no sex predilection. The marriages which resulted in afflicted persons were all consanguineous, but in no case was a parent afflicted. The inheritance ratio was somewhat in excess of the expected 1:3, in that there were 10 afflicted

siblings and 21 normal children in four sibships but Hutchison and McGirr point out that undoubtedly a number of unaffected siblings were lost to genetic study [148]. To date there have been no marriages of the afflicted members of this kindred so that there has been no opportunity to test inheritance from phenotypes.

It seems a reasonable assumption that the cases of cretinism with goiter described from the Netherlands and from Scotland are homozygous. The defect observed in these patients was quite complete. There was no evidence whatsoever of any deiodination of DIT. On the other hand five relatives of one of the Netherlands cases demonstrated defective DIT deiodinase activity and all had evidence of thyroid disease but without mental or skeletal retardation. It follows that these relatives represent the heterozygous condition [149]. It is provocative that one of the patients in this kindred had thyrotoxicosis and another had died with a diagnosis of thyrotoxicosis but a relationship between the deiodinase defect and thyrotoxicosis is not apparent.

If as seems probable the gene which is defective in these patients is responsible for the production of iodotyrosine deiodinase then it might be supposed that in the heterozygous condition enough of the deiodinating enzyme would be produced to handle normal loads of the metabolite particularly if through thyroid hypertrophy enough hormone precursor could be retained in the thyroid to satisfy the demands of iodothyronine synthesis. If this is the case it should be possible to test the deiodinase activity in heterozygotes by challenging the patient with an intravenous dose of labeled DIT to which an amount of carrier DIT was added which would overload the deiodinating capacity of the limited enzymatic endowment of the patient. There has been no opportunity to test this hypothesis but if a normal individual is given between 25 and 50 mg labeled DIT then approximately 40 to 50 per cent of the labeled iodine appears in the urine as unchanged DIT. If such a test could be performed on the goitrous but euthyroid relatives of a patient with full deiodinase defect cretinism and goiter a far higher fraction of the administered DIT might appear unchanged in the urine than in normal individuals. If this hypothesis is proved true a method will be provided for detecting the carrier state of the deiodinase defect.

#### CRETINISM WITH IODINATED POLYPEPTIDES IN THE SERUM

The final category of patients with goiter and hypothyroidism which will be discussed in this chapter is less well defined and the biochemical mechanisms less well perceived than some of those which have already been discussed. The patients are characterized by the appearance in the blood of iodinated amino acids which are in peptide linkage and which are not extractable into acid butanol as are the iodinated thyronines which normally appear in the peripheral blood.

### Case Studies

The first of these patients to come to the authors' attention were two sisters in the clinic of McGirr at the Royal Infirmary in Glasgow [2]. Both were in their early twenties, were retarded mentally, were hypothyroid and had large nodular goiters. Each had been given a tracer of  $I^{131}$ , and samples of serum had been obtained serially for many hours. The peculiarity of the samples was that the labeled iodine of the serum was incompletely extractable into butanol. When the samples were acidified to pH 2 it was possible to extract only 50 to 60 per cent, whereas 85 to 95 per cent is readily extractable from normal serums. These studies were not pursued further at that time.

In 1956 Whitelaw, Thomas, and Reilly reported in abstract an 8½-year-old female cretin with a PBI concentration of 11.2 and 11.3 µg per 100 ml [159]. There was no goiter. The 24-hr  $I^{131}$  uptake approached 100 per cent. Chromatographic analyses of samples of serum—performed in the laboratory of Werner, Block, and Mandl [160]—disclosed a variety of iodinated compounds, but it was of particular interest that in three separate samples a large fraction of the labeled iodine was in the form of an unidentified iodoprotein.

In the following year DeGeorge and Pashkin described detailed studies on a 7½-year-old retarded female who had a high uptake and retention of  $I^{131}$  [161]. There was a small goiter. The serum concentration of PBI was 7.7 and 9.6 µg per 100 ml. Chromatographic analyses were performed by J. Gross. In two successive samples of serum 85 and 63 per cent of the labeled iodine failed to extract into butanol. That which did extract was identified as  $T_4$ . The authors suggested that a calorigenically inefficient product was being secreted by the gland and that it was an iodoprotein. In an addendum they briefly reported another similar case.

A short while later Werner et al. [160] reported a 40-year-old female with signs and symptoms suggestive of hypothyroidism but without goiter. Protein-bound iodine was 4.2 µg per 100 ml, and the 24-hr  $I^{131}$  uptake was 25 per cent. Approximately one-third of the labeled iodine in the serum failed to extract into butanol. Upon hydrolysis in the presence of strong acid MIT and DIT were reported in chromatograms of serum samples.

Several patients with an abnormal iodinated component in their serums which is poorly soluble in butanol have been studied by DeGroot et al. in the author's Laboratory [162, 163].

**Patient 1.** This patient was a 28-year-old cretinous female with short stature and imbecility. A large nodular goiter which was removed 10 years previously had shown hyperplasia on histologic examination. There was moderate regrowth of the thyroid when thyroid medication was discontinued several weeks prior to study in 1957.

The significant findings were a high uptake of  $I^{131}$  and an iodinated fraction in the serum which was not extractable into butanol when the serum was acidified to pH 2. Only inorganic iodide was demonstrable in the urine. Deiodination of administered labeled DIT was normal, and there was no discharge of labeled iodide from the gland after administration of  $KSCN$ . The half time of retention of labeled iodine in the gland was 18 days. The half time of retention of administered labeled  $T_4$  was 5.5 days (normal).

Particular interest centered on the abnormal iodinated component in the serum. Extractability into butanol was increased by treatment of the serum

with proteolytic enzymes such as trypsin chymotrypsin or pepsin. Chromatograms of these serum hydrolyzates revealed MIT and DIT in addition to iodide  $T_4$  and  $T_3$ .

The abnormal iodinated component of the serum was transported differently from  $T_4$ . When the serum was subjected to electrophoretic analysis in a starch supported barbital buffer at pH 8.6 the principal iodinated component was found as a single zone associated with the peak and the advanced limb of the albumin fraction (Fig 9-12). Although this component had an

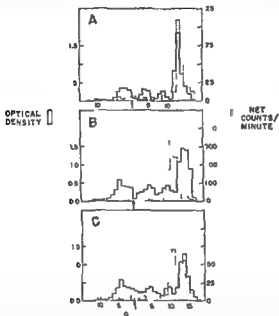


Fig 9-12 Starch block electrophoresis of serum of a patient with cretinism and goiter (see text). A: Four days after administration of  $I^{131}$ . B: 12 hr after  $I^{131}$  labeled  $T_4$  administered intravenously. C: 4 days after a second dose of  $I^{131}$ . The shaded area is labeled iodine. The open areas correspond to protein. Note the presence of a labeled compound corresponding to albumin in A and C and in B a failure of T to conform to albumin or the in vivo labeled iodine. (By permission from *The Journal of Clinical Endocrinology and Metabolism*.)

electrophoretic mobility of normal human serum albumin it could not be precipitated with an antiserum against normal serum albumin produced in the horse.

Thyroxine was found in its usual position in the interalpha zone where it was presumably in association with the normal T binding protein (Fig 9-12). When  $I^{131}$  labeled  $T_4$  was added to the serum of this patient or to the serum of normal patients it proved to be more than 90 per cent extractable under conditions which gave only a 60 or 65 per cent extractability of the iodinated components of the patient's serum labeled in vivo. Thus there was no abnormality of the thyroid binding protein of the serum.

**Patient 2:** A 10-year-old female with retarded mental and physical development and a goiter was studied 2 months after discontinuance of medi-



cation with desiccated thyroid. An  $I^{131}$  uptake was 66 per cent in 2 hr and 71 per cent in 24 hr. The half time of the labeled iodine in the gland was 12 days. There was no release of labeled iodine from the gland by ASCN. Approximately half the labeled iodine of the acidified serum was insoluble in butanol. Sixty-three per cent traveled with albumin on starch block electrophoresis. The butanol solubility of the labeled iodine of the serum was increased to 91 per cent following enzymatic digestion. Both iodide and  $T_4$  were present in chromatograms of butanol extracts of the serum and labeled components with the mobility of MIT and DIT and of iodothyronines were present in chromatograms of samples of urine. Twenty per cent of the labeled iodine in the urine was not soluble in butanol.

*Patient 3.* A 42-year-old mentally dull male was known to have had a goiter since age 14. It was removed at age 23 but subsequently recurred. A mother and sister had goiter. There was a 60 per cent uptake of labeled iodine at 2 hr and 82 per cent in 24 hr. The half time of the labeled iodine in the gland was 145 days. The concentration of the labeled iodine in the serum was only 0.05 per cent per liter at 48 hr. Of this 24 per cent was not extractable into butanol. The extractability was increased to 90 per cent by digestion with chymotrypsin. Chromatograms of the serum revealed only iodide and  $T_4$  and  $T_3$ . Chromatography of the urine disclosed only iodide.

Detailed studies were performed on thyroid tissue received at the time of subtotal thyroidectomy. Deiodination of MIT by gland homogenate was normal. Chromatographic analysis for the iodinated amino acids after enzymatic digestion disclosed a normal distribution except for a somewhat reduced amount of  $T_3$  and  $T_4$ . Electrophoretic analysis of a saline extract of this gland showed two major components, one of which occupied the electrophoretic position of normal thyroglobulin. The other had the mobility of serum albumin and contained no  $I^{131}$ . Preparative electrophoresis on a paper curtain failed to resolve this prethyroglobulin component. Ultracentrifugal analysis of the  $I^{131}$ -containing peak from the preparative electrophoresis revealed material which had a low sedimentation constant. There was not only a zone of normal thyroglobulin with an  $S_{20,w} = 19$  but a much larger component with an  $S_{20,w} = 4$ . The  $I^{131}$  protein in the thyroid saline extract was salted out of solution by 2 to 2.3  $M$  phosphate buffer.

*Patient 4.* A 14-year-old schoolgirl with normal development was found to have had a goiter at age 4. The thyroid was four times enlarged. The PBI concentration of the serum was  $3 \mu g$  per 100 ml.  $I^{131}$  uptake was 50 per cent in 1 hr and 85 per cent in 24 hr. The half time of retention in the gland was 83 days. The serum  $I^{131}$  concentration at 48 hr was 0.44 per cent per liter and at that time 30 per cent of the PBI $^{131}$  was not extractable into butanol. Chymotrypsin digestion increased the extractability to 93 to 95 per cent. Fifteen per cent of the labeled iodine in the urine was not extracted by butanol but all was dialyzable. The iodinated component of the urine was not identified but in a butanol-acetic acid solvent system it remained close to the origin.

A number of studies were done on a biopsy specimen of the thyroid. A whole homogenate deiodinated MIT and DIT. No thyroglobulin was found. On electrophoretograms the labeled iodine was found in a zone close to the origin in the region which in serum is occupied by the  $\beta$  gamma globulins. Ultracentrifugal analysis showed only one peak at  $S_{20,w} = 4$ . Salting out with phosphate buffer was abnormal. There was no sharp separation with increasing salt concentration but rather a gradual salting out between 2 and 2.5  $M$ .

*Patients 5 and 6* Samples of serum and urine were received from H Courvoisier of Geneva, Switzerland. They were obtained from two male siblings age 15 and 17 who had been hypothyroid and goitrous during most of their lives. The PBI of the first of these was 5.2 and the  $I^{131}$  uptake at 24 hr was 90 per cent. The serum at 72 hr contained 0.69 per cent of the dose per liter. Approximately 60 per cent of the PBI<sup>131</sup> was not extractable into butanol. Thyroxine,  $T_4$ , and iodide, MIT and DIT, were identified in the serum and possibly small amounts of MIT and DIT. The butanol extractability of the serum  $I^{131}$  was increased by chymotrypsin digestion. The butanol insoluble component traveled with albumin on paper electrophoresis. It did not salt out with phosphate buffer at concentrations below 2.2 M. Substances were found in the serums which had the  $R_f$  of iodide, MIT and DIT in the butanol-acetic acid solvent system. There was also an unidentified component with an  $R_f$  less than iodide.

The PBI of the brother was 1.5  $\mu$ g per 100 ml and the  $I^{131}$  uptake was 84 per cent. At 96 hr the serum contained only 0.1 per cent of the dose per liter. Only iodide was identified with certainty in the serum but the concentration of labeled iodine was too low for satisfactory studies. Thirty-five per cent of the PBI<sup>131</sup> was not extractable into butanol. A nonbutanol extractable component was present in a concentration of 15 per cent in the urine collected on the fourth day.

A retarded patient, the product of a first-cousin marriage, has recently been reported in detail [163a]. Observations very similar to those on Patient 1 above were obtained. The labeled iodine in the serum was poorly extractable into butanol and on electrophoresis moved with albumin.

### *Physiological Interpretation*

The differences observed among the patients of this category may possibly be attributable to variations existing among patients who have in common certain fundamental abnormal characteristics. All showed evidence of hypothyroidism and all had goiter which appeared at an early age. All had in addition an iodinated component in the peripheral blood which was identified by labeling with  $I^{131}$  and which was not extractable from the acidified serum into *n*-butanol. Three had an unidentified iodinated component in the urine which was not extractable into butanol and which was dialyzable. The other two did not have this finding. One of the patients whose thyroid tissue was available had no thyroglobulin and the other had an increased amount of a component with an  $S_{20,0} = 4$ . Two of the patients had iodinated components in the peripheral blood which had the chromatographic characteristics in the butanol-acetic acid solvent system of MIT and DIT.

The nature of the circulating iodinated component in the peripheral blood was of particular interest. Upon hydrolysis with trypsin or chymotrypsin, iodinated MIT, DIT,  $T_3$  and  $T_4$  were released and the butanol solubility of the labeled iodine was increased.

The data presently available do not permit an exact interpretation of the abnormality. The thyroid is secreting an abnormal iodinated component into the blood which is calorimetrically ineffective. This com-

ponent is hydrolyzable with crude trypsin, chymotrypsin, and pepsin to release iodotyrosines and iodothyronines.

The significant question which cannot be answered at present regards the reason for the production and secretion of an abnormal polypeptide. Such production could conceivably be due to an abnormality in the synthesis of thyroglobulin (i.e. the formation of an abnormal thyroglobulin in a way analogous to genetically controlled synthesis of abnormal hemoglobins) or to defective proteolytic degradation of thyroglobulin because of either an altered nature of the thyroglobulin itself, or a defect in one of the proteolytic enzymes of thyroglobulin degradation. At present it is impossible to exclude any of these possibilities and indeed all may be operative in different groups of patients. Before a final answer can be given it will be necessary to search for abnormal thyroglobulins and for abnormalities in the enzymatic components of the stepwise scheme of thyroglobulin proteolysis.

### Genetics

Too few patients have been studied for the genetics of this disease or group of diseases to be characterized. The two sisters in Dr. McCurr's clinic and the two brothers in the clinic of Dr. Courvoisier strongly suggest that the disease is hereditary. In so far as is known, no affected parents have been found in the kindred of any one of the 11 patients except that the mother of one had an asymptomatic goiter.

## DIAGNOSIS

There are no distinguishing clinical features among the various types of familial goiter with hypothyroidism except for that group which has an associated nerve deafness. Differential diagnosis of the various categories which have been described must in general depend upon specific laboratory tests [154]. There is sufficient information already available on many patients described in the literature on which to base a retrospective presumptive diagnosis as has been seen. This is not possible for several interesting case reports which have not been mentioned because of insufficient data [155-159].

The most useful preliminary observation is the uptake and retention of labeled iodine by the thyroid gland. Characteristically the 11 patients have an unusually rapid uptake curve which reaches high levels within the first hour or two following administration. Patients with the organification defect lose the labeled iodine from the gland rapidly after the peak uptake value is reached and this rate of loss may be made precipitous by the administration of perchlorate or thiocyanate. The same is true of those patients with associated congenital goiter and nerve deafness. Diagnosis is made certain if it can be shown that there is no organically bound iodine in the removed gland.

Patients who have the iodotyrosine coupling defect also have a high rate of uptake of the labeled iodine but it is retained in the gland and disappears with only a moderate acceleration of the normal half time. The diagnosis in these patients can be made with reasonable certainty only by showing that a specimen of thyroid tissue removed 72 hr or later following administration of labeled iodine contains an abundance of labeled MIT and DIT but no detectable labeled  $T_4$  or hardly any. No abnormal iodinated substances should be present in the blood of such a patient.

Patients with the deiodinase defect also have a rapid uptake phase following administration of labeled iodine. There is rapid loss of labeled iodine from the gland after the peak uptake value is reached followed by a slower loss beginning 24 hr or so after administration. A few patients who undoubtedly belong to this group do not have this phase of rapid release. Chromatographic analysis of blood and urine discloses the presence of iodinated substances which have the chromatographic mobility of MIT and DIT and their derivatives and conjugates. The final test is to show that an administered dose of labeled DIT is excreted almost or entirely unchanged in the urine.

Patients with congenital goiter and hypothyroidism who have butanol insoluble iodinated components in their serums have normal or elevated retention curves of labeled iodine. Diagnosis depends upon demonstration in the acidified serum of butanol unextractable iodine and the further demonstration that proteolytic hydrolysis of this component yields the usual iodinated thyronines and tyrosines. It seems entirely possible that this group is heterogeneous and that with further study it will be subdivided into several groups according to specific abnormalities of the synthetic and degradative schemes for thyroglobulin in the thyroid gland.

The discussion thus far has centered on the diagnosis in patients who have a complete defect, i.e. homozygous expression. An important problem and a more difficult one from many points of view is the diagnosis of a specific genetic defect in patients who may be heterozygous for that defect. From studies done to date it is clear only in the deiodinase defect group that the heterozygous state may cause disease. In one case it was possible to show that various goitrous members of the family of a patient with fully expressed cretinism and deiodinase defect were less able to deiodinate injected DIT than normal persons. More recent studies have shown that increasing the dose of DIT to approximately 20 mg significantly loads the deiodinating system in vivo in normal man [160]. It is quite possible that this load might completely overwhelm the deiodinating system in a subject with the deiodinase defect in the heterozygous state.

There is now evidence that certain patients with nodular goiter but without evidence of present or past hypothyroidism may have small but

detectable amounts of nonbutanol-extractable iodine in their serums [103]. It remains to be shown that these patients are heterozygous in relation to patients homozygous for the same defect who have congenital goiter and hypothyroidism. Unfortunately in these patients the glands are so filled with iodine and so sluggish in their activity that the turnover of labeled iodine is extremely low. Manipulations with the small amounts of labeled iodine appearing in the serum after tracer doses are difficult to the point of uncertainty.

## TREATMENT

Satisfactory treatment of familial goiter depends upon the stage of development of the local disease and the degree to which irreversible changes have occurred in the skeleton and central nervous system. Remarkable shrinkage of the goiter may be expected from treatment with L-thyroxine or desiccated thyroid in usual maintenance doses providing irreversible changes of degeneration, cyst formation and fibrous replacement have not taken place. The goiter will inevitably recur if medication is discontinued.

Care should be exercised in treating a familial goiter in view of the tendency of some of them to undergo malignant change. In general it would be wise to remove any nodule which fails to shrink after several weeks of replacement therapy. Unfortunately this will often be the case with well-established goiters so that more often than not these patients eventually require surgery. Desiccated thyroid is required after thyroidectomy unless it is desirable to maintain the patient in a myxedematous state.

Unless treatment is begun early within the first few weeks of life there is considerable risk of permanent retardation of intellectual development or skeletal growth. There is reason to suspect that damage may occur in utero so that no amount of replacement therapy will prevent developmental arrest and permanent retardation.

Medication should be sufficient. Perhaps a safe rule is to give increasing doses of desiccated thyroid until the first signs of overdosage appear (tachycardia, hyperactivity) and then reduce the dosage slightly. Particular attention should be paid to newborn siblings of patients who have familial goiter. Carr et al. [161] have recently reported a mother who produced an apparently normal infant after two successive pregnancies by taking large doses of desiccated thyroid during pregnancy. The child was later shown to have no thyroid gland. Presumably the large doses caused sufficient placental transfer of hormone to permit normal development of the fetus.

Management of the patient with fully developed goiter, hypothyroidism and permanent retardation is unsatisfactory. Little is accomplished in the adult by replacement therapy and as often as not inac-

ceptable aggressiveness or other undesirable behavior may be the result of full thyroid medication. In these patients the dosage is best adjusted to that which keeps the patient active and comfortable without arousing unwanted side effects.

A few patients with familial goiter such as some of those who have associated eighth nerve deafness appear to manufacture adequate amounts of hormone and to develop normally. They require thyroid substance only to prevent growth of the small goiters to which they are predisposed.

## SUMMARY

1 The synthesis, storage, secretion, delivery, and utilization of the thyroid hormones involve a complex sequence of metabolic events, each of which is probably dependent upon specific enzymatic activity. Thyroid disease may result from blockage at many steps in this metabolic process.

2 Familial goiter and hypothyroidism may occur when there is failure to convert inorganic iodide into iodine in the thyroid gland. Accumulated iodide is precipitously discharged from the gland upon the administration of thiocyanate.

3 A group of patients possibly closely related to the above have goiter and congenital nerve deafness. Labeled iodine in the gland is partially discharged upon the administration of thiocyanate.

4 Three families of patients have now been discerned who have a defect in the coupling of iodotyrosines into iodothyronines. The disease can be demonstrated with certainty only when it is shown that iodothyronines fail to appear in biopsy specimens of thyroid tissue.

5 Several family groups are known to be unable to deiodinate iodotyrosines. Loss of hormone precursors from the gland and into the urine accounts for hypothyroidism and compensatory goiter. Several goitrous but otherwise normal relatives of one such patient have been shown to deiodinate diiodotyrosine less well than normal individuals. The condition is most convincingly diagnosed by the demonstration that intravenously administered labeled DIT is excreted intact in the urine.

6 Certain patients with congenital goiter have been found with a circulating butanol-insoluble iodinated component in the serum. Certain slight differences among patients suggest that this group may be heterogeneous. The disease may represent a defect in thyroglobulin synthesis or proteolytic degradation in the thyroid gland.

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## Chapter 10

### Phenylketonuria\*

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by Eugene Knox

The study of phenylpyruvic amentia may throw light on the whole problem of mental deficiency. [1] Now only 25 years after Fölling's original description of phenylketonuria [1] this modest statement by the leading investigator of this disease points out the sole major problem left to be solved. This chapter aims to collect and codify the considerable knowledge of phenylketonuria in the hope that it will serve as a basis primarily for the future study of the mental defect in phenylketonuria, an endeavor which can potentially furnish insight into the nature and mechanism of development of the intellectual functions.

#### HISTORY AND DEFINITION

Garrod's recognition of the relationship between gene, enzyme, and clinical abnormality 20 years before the first case of phenylketonuria was described provided the conceptual basis for the disorder [2]. Indeed it was the first of these diseases to have the postulates of Garrod unequivocally demonstrated.

In 1934 Fölling described 10 patients, some of them siblings, who excreted phenylpyruvic acid and were mentally deficient. Jervis [3] then proved that the condition was inherited through a single autosomal recessive gene and showed that large amounts of phenylalanine accumulated in the body of these patients [4]. He located the metabolic error as the inability to oxidize phenylalanine to tyrosine [5]. In 1953 Jervis demonstrated that the phenylalanine hydroxylase of the liver was inactive in these patients [6]. A rational therapy, better described as an effective preventive regimen consisting of a low phenylalanine diet, was then developed [7, 8]. Tests for distinguishing the heterozygotes of phenyl

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ketonuria followed [9]. These salient facts provide the means for understanding and controlling phenylketonuria. Many cogent reviews of this disease have appeared [10-15]. It has been variously termed *imbecillitas phenylpyruvica* [7], *Fölling's disease* and *phenylpyruvic oligophrenia* [7]. The term *phenylketonuria* first introduced by Penrose and Quastel [16] has now been generally adopted.

The uniform manifestations and precision with which phenylketonuria can be identified allow it to be defined exactly. It is required that both parents of a patient have the defective form of one of the two genes controlling phenylalanine hydroxylase. An apparent reduction in phenylalanine hydroxylase activity can be detected in the parents but there is no serious consequence of this inadequacy. On the average one out of four offspring from two heterozygous parents has both genes defective; in these offspring there is no active phenylalanine hydroxylase and phenylketonuria results. Both sexes are affected equally often.

The disability is first manifest several weeks after birth initially by elevation of plasma phenylalanine to thirty times the normal level and by the excretion of phenylpyruvic acid. After 6 months retardation of mental development is evident. Seizures and other neurologic abnormalities, diluted pigmentation of hair and skin and eczema may occur. In older children and adults the process remains stationary but life expectancy is reduced. The majority of patients are idiots, a few are imbeciles and rare patients have borderline intellectual development.

The incidence of the disease is approximately 1 in 25,000 among mixed populations stemming from northern European countries. Most of the patients are found in institutions where they make up about 1 per cent of the mentally defective population. The disease is readily diagnosed by the olive green color produced in the urine upon addition of  $\text{FeCl}_3$ . This color is due to phenylpyruvic acid.

## CHARACTERISTICS

### INCIDENCE

The primary data for calculating the incidence of phenylketonuria consist of its frequency among institutionalized mentally defective individuals. Surveys of a total of 48,536 defective patients examined in 12 different countries were compiled by Jervis [12]. A total of 312 phenylketonuric patients was found or an average incidence of 0.64 per cent of the world wide institutionalized defective population. The problems raised by these data are whether the disease is evenly distributed among peoples and the numerical relation between the defective populations and the populations from which they are drawn. The incidence in single institutions varied from 0 per cent in French Canada to 2.7 per cent in England. From the figure of 0.64 per cent as the incidence of phenyl

ketonuria in the defective population and an estimate of somewhat less than 1 per cent as the incidence of defectives in the general population (estimated for the United States), Jervis calculated that the incidence of phenylketonuria in the general population was between 2 and 6 per 100 000. He accepted 4 per 100 000 as the order of the real figure. It is clear that better estimates of the incidence could be obtained only for specific populations.

Other local and independent estimates of the incidence of phenylketonuria are therefore of interest. Munro [17] calculated the incidence in England to be between 2 and 6 per 100 000. For a south Swedish region comprising nearly half the Swedish population, Larsson [18] similarly calculated an incidence of 3.5 per 100 000. There were first-cousin marriages among 12.5 per cent of the parents of the Swedish phenylketonuria probands. From Larsson's [19] later estimate of 1.7 per cent frequency of first cousin marriages in this population, a similar incidence of phenylketonuria can be calculated.

Methods which approach complete ascertainment in localized populations have given closely similar figures. A.C. Stevenson's unpublished data for the incidence in Northern Ireland were 10 per 100 000 at birth and 3 per 100 000 in the living population. Armstrong and Low [20] calculated an incidence of 1 in 20 400 in Utah. They noted that all except 3 of the 18 phenylketonuric persons born in Utah and known to them were under 16 years of age. They suspected that this unusual age distribution might be due to a high death rate among phenylketonuric patients. It should be noted that Larsson's [18] estimate of the incidence of phenylketonuria in Sweden included a correction for the higher mortality among low grade defectives. It can be concluded that a varying incidence of from 3 to 5 per 100 000 occurs in populations of North European origin.

The survey of Jervis [12] indicated that phenylketonuria was most common in Northern European or Scandinavian countries and in populations derived from this stock. The average was heavily weighted by the high proportion of surveys from such countries. The incidences therefore hold only for countries of this population make-up. In individual series of case from such countries as the United States, it was found that certain segments of the population contributed far fewer phenylketonuric persons than expected from their numerical representation in the general population. Jervis found no phenylketonuric patients among 1 000 mentally defective Jews, and only one whose parents were Negro-Indian and Negro-White. Jews and Negroes were common among the mentally defective population studied Italian, on the other hand, made up about 18 per cent of both the phenylketonuric patients and the institutional population studied [21]. The handful of exceptional cases not of Nordic origin which have been reported are tabulated in Table 10.1. These



cases indicate that the disease is exceptionally rare but is not unknown in Jewish, Negro and Japanese populations. Two patients described by Bhaskaran [28] in the *Indian Medical Gazette* were Canadians.

It should be noted that an incidence of phenylketonuria of about one in 25 000 in the general population is of the same order of magnitude as that of other rare inborn errors of metabolism like alcaptonuria, cystinuria and albinism. Yet the number of cases reported in only 20 years was 33 in 1951 [12]. With the interest aroused since then by treatment of this

TABLE 10-1 NON-NEURIC CASES OF PHENYLKETONURIA

| Origin       | No. of individuals | Reference    |
|--------------|--------------------|--------------|
| Spanish      | 1                  | [1]          |
| Jewish       | -                  | [3]          |
| Mexican      | 1                  | [17]         |
| Negro        |                    |              |
| Indian-white | 1                  | [9] (see 99) |
| Brazilian    | 1                  | [4]          |
| Mulatto      | 1                  | [1]          |
| Japanese     | 8                  | [1]          |

disease the total of reported cases is approaching 1 000. This is a considerably larger number than the total cases reported over many years of the other rare hereditary diseases.

Larson's estimate of 3.5 phenylketonuric persons per 100 000 include 1.1 per 100 000 drawn with relatively high incidence (0.76 per cent) from idiots and imbeciles (who make up only 0.15 per cent of the population) and 2.4 per 100 000 drawn with relatively low incidence (0.16 per cent) from the more frequent morons in the population (1.5 per cent). Since the latter group is incompletely institutionalized and since it contributes the largest absolute number of phenylketonuric patients there may be many noninstitutionalized persons with phenylketonuria. A study of 8 220 individuals not known to be mentally defective did not reveal any cases of phenylketonuria [29] nor did a survey of some 10 000 young healthy men in military service [12]. But among morons Larson [18] found 2 in 1 277, and Schonenberg et al. [30] 1 in 2 016. Gibbs and Woolf [30a] found 1 in a survey of 1 141 normal infants. Although a large absolute number of unrecognized phenylketonuric persons might be expected in the general population they would be so rare (<2.4 per 100 000) that only enormous surveys could find them. It would appear from the available figures that the absolute number of known cases of phenylketonuria could perhaps be doubled by recognition of all cases with higher grade intellectual function.

## AGE DISTRIBUTION AND CAUSE OF DEATH

The age distributions of all sizable series of patients show deficiencies in the numbers of those under 5 years and of those over 35 years of age. The former reflects tardy diagnosis plus a period when patients may be kept at home since the majority of reports come from institutions. The scarcity of older patients may be only the result of the higher death rate common to institutionalized persons and particularly to those who are mentally defective. The graphed age incidence curves of phenylketonuric

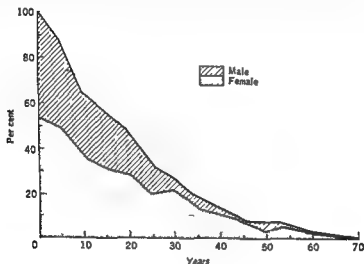


Fig 10-1 Age and sex distribution of phenylketonuric patients (By permission of A. Laq [19])

patients constructed by Lang [13] from the ages of 500 reported patients (Fig 10-1) indicate that half died by 20 years of age and three quarters by 30 years of age. A group of 34 patients from Massachusetts included 2 over 50 years and 2 who were 60 years of age [31]. Alvord et al [32] performed an autopsy on a 67-year-old patient. Lang [13] referred to a 68-year-old patient. Hassel and Brunsting [32a] studied a 72-year-old patient. There has been no study to find if the life span of phenylketonuric patients is shortened in a specific way except for Lang's evidence that males die earlier than females.

The ages and causes of death of 26 patients are available from the autopsy reports given in Table 10-2 plus a few others mentioned casually in the literature [6, 10, 31-38]. These are summarized in Table 10-3. Most of the reported deaths were in young individuals. The majority died from infectious diseases that are particularly prevalent among institutionalized populations.

TABLE 10-2 PATHOLOGIC ANATOMY OF PHENYLKETONURIA—AUTOPSY LIST

| Age yr | Sex | Mental state      | Cause of death                                 | Central nervous system  | Reference |
|--------|-----|-------------------|--|---|-----------|
| 14½    | M   | Severely retarded | Staphylococcal septicemia 1 yr illness         | Marked lack of myelination  | [39]      |
| 13½    | F   | Severely retarded | Vomiting and epileptic crisis old tuberculosis | Lack of myelination extensive gliosis   | [33]      |
| 3      | M   |                   |  | Paleness of myelin in temporal regions  | [12]      |
| 5½     | F   | Idiot             | Interstitial pneumonia                         | Areas of deficient myelination  | [39]      |
| 9½     | M   | Idiot             | Bronchopneumonia                               | Cerebral edema  | [31]      |
| 12     | F   | Idiot             | Cardiac pyrexia liver degeneration             | One small necrotic spot in frontal white matter                                 | [30]      |
| 14     |     |                   |  | Hypopigmented basal ganglia   | [36]      |
| 15     | M   | Idiot             | Intestinal obstruction                         | Slight paleness of myelin in corticopontine tracts and hyperplasia of macroglia | [31]      |
| 18     | F   | Idiot             | Bronchopneumonia liver disease                 | No gross pathologic change  | [33]      |
| 20     | F   | Idiot             | Perforated ventricular ulcer                   | Dense cytoarchitecture in deepest cortical layers                               | [31]      |
| 21     | F   | Idiot             | Pulmonary and abdominal tuberculous            | No significant histologic changes   | [31]      |
| 21     | F   | I Q 8             | Pulmonary tuberculosis                         | Degeneration of white matter throughout brain                                   | [10]      |
| 21     | M   | I Q 21            | Pulmonary tuberculosis                         | Degeneration of myelin (pons cerebellum and cord) and deficiency elsewhere      | [10]      |
| 21     | F   |                   | Cavernous pulmonary tuberculosis               | Large medullary cyst (artifact?)  | [41]      |
| 25     | F   | Idiot             | Pneumonia                                      | No gross pathologic change  | [33]      |
| 25     | F   | Idiot             | Fever—5 days                                   | Intense gliosis of occipital lobes  | [19]      |
| 25     |     | Imbecile(?)       | Schulder's disease                             | Typical lesions   | [19]      |
| Adult  |     |                   |  | No demyelination  | [12]      |
| Adult  |     | Idiot             | Pulmonary tuberculosis                         | No significant abnormality  | [3]       |
| Adult  |     | Idiot             | Pneumonia                                      | No significant abnormality  | [43]      |
| Adult  |     |                   | Biliary tuberculosis                           | Sieve like extrapyramidal centers   | [44]      |
| Adult  |     |                   |  | No gross pathologic change brain weight low                                     | [45]      |
| 33     | M   | Idiot             | Pulmonary tuberculosis                         | Honeycombed center of optic chiasma myelin loss and gliosis                     | [39]      |
| 51     |     |                   | Thyroid carcinoma                              | Glial excess—astrocytes   | [31]      |
| 67     |     |                   | Brain biopsy                                   | Glial excess—astrocytes   | [39]      |
|        |     |                   | Brain biopsy                                   | Normal cells and tissues  | [46]      |
|        |     |                   | Brain biopsy                                   | Normal cells and tissues  | [16]      |

TABLE 10-3 CAUSES AND DECADE OF DEATH IN PHENYLKETONURIA

| <i>Decade</i> | <i>No of deaths</i> | <i>Cause</i>         | <i>No of deaths</i> |
|---------------|---------------------|----------------------|---------------------|
| 1             | 8                   | Tuberculosis         | 8                   |
| 2             | 4                   | Pneumonia            | 6                   |
| 3             | 10                  | Liver disease        | 3                   |
| 4             | 1                   | Nephritis            | 3                   |
| 5             | 0                   | Perforated ulcer     | 2                   |
| 6             | 2                   | Septicemia           | 1                   |
| 7             | 1                   | Carcinoma            | 1                   |
|               |                     | Miscellaneous 1 each | 2                   |
| Total         | 26                  | Total                | 26                  |

## SLA

The difference in death rates probably is the reason why numerous small series in the literature have shown a slight excess of female over male patients. There was no difference between the sexes in the massive compilation by Jervis [12]: 237 females (51 per cent) and 228 males (49 per cent). The characteristics of the disease in the two sexes have not been compared.

## INTELLIGENCE

*Deterioration*

Patients with phenylketonuria not infrequently lose accomplishments they once had in childhood. This should not be interpreted as evidence for a progressive disease process after infancy. Paine [47] had the following to say about this question:

Cessation of independent locomotion however is by no means rare in very low grade retarded patients and it is quite common for very retarded children at one stage to reproduce a parrot like speech which is later lost. This repeating of words spoken to the child by parents is perhaps more akin to a conditioned trick such as playing pat-a-cake or waving goodbye and may be lost if it is not later converted into the use of words to convey meaning rather than merely to win approval.

He also mentions that early estimates of intelligence inevitably depend on motor development which is likely to be less delayed than the acquisition of speech and other interests or abilities after infancy. A young patient therefore appears proportionately less retarded in infancy than in later childhood. Institutionalization itself can also account for the apparent deterioration of some patients. Others have concurrent organic brain disease (see Pathologic Findings below and Table 10-2). Nevertheless a real deterioration magnified by the failure of expected capabilities

to develop often occurs shortly after 6 months of age when the severe neurologic disturbances appear

### *Distribution*

The intelligence quotients of phenylketonuric patients in two independent studies of institutionalized patients are shown in Fig 10-2 [12, 47]. The great majority are idiots ( $IQ < 20$ ) and the remainder nearly all imbeciles ( $IQ < 50$ ). Many cannot walk, talk, or control their excretory sphincters.

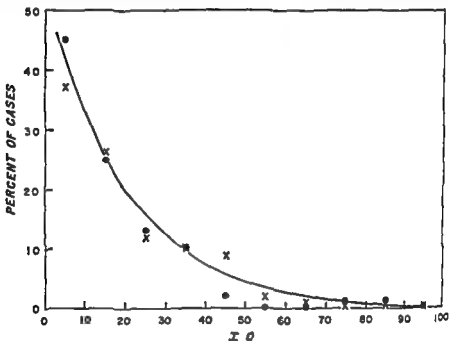


Fig 10-2 Frequency distribution of  $IQ$ 's among 370 patients [7] and 101 patients [51]. The total of 2 per cent above  $IQ$  60 would account for 20 such patients in a total of 1,000. Table 10-4 lists the 16 such patients who are known.

Lang [13] noted that the intelligence of phenylketonuric patients over the age of 10 years is higher on the average than at younger ages because of the earlier deaths of the most severely affected ones. Larson's examination of 1,277 institutionalized morons revealed only 2 phenylketonuric patients (0.16 per cent) as compared with a fivefold higher incidence among idiots and imbeciles (0.76 per cent). Schönberg et al [30] found only 1 phenylketonuric patient among 2,016 students in special schools for morons. Woolf and Vulliamy—cited in [49]—found only 1 case among 2,200 educationally subnormal children ( $IQ$  50 to 70). The surveys total 4 in 5,493 subnormal persons (0.07 per cent) and establish that the

general shape of the distribution curve is not an artifact arising simply from more frequent hospitalization of the more severe defectives. The trend of the I Q distribution curve of known cases emphasizes that high grade defectives must be rare. Probably less than 2 per cent of all phenylketonuric patients would be expected to have I Q's above 60.

### High-grade Phenylketonuric Mental Defectives

The subtlety of the factors making for the intelligence of the human mind is nowhere better shown than in the occurrence of these few reasonably competent patients with a disease that almost invariably produces idiots or imbeciles. Their existence is the best argument against the term 'phenylpyruvic oligophrenia'. Although none of the high grade cases reported is intellectually normal, their superiority to the vast majority of phenylketonuric patients is most interesting.

A total of 16 high grade (intellectually 'borderline' or 'dull and backward') phenylketonuric patients has been reported. They are listed in Table 10-4. To these perhaps should be added Cases 31 and 34 of Fölling

TABLE 10-4 HIGH GRADE UNTREATED PHENYLKETONURIC PATIENTS

| No | Age | Sex | I Q | Phenylpyruvic acid (urine)                           | Phenylalanine (plasma) $\mu$ moles/ml | Reference |
|----|-----|-----|-----|--|---------------------------------------|-----------|
| 1  | 6½  | F   | 87  | 0.8-1.5 gm/gm creatinine                             | 1.15-1.88                             | [50]      |
| 2  | 3   | F   | 91  | Positive   |                                       | [51]      |
| 3  | 6   | F   | 91  | 0.016% gm/hr   | 0.03 (average of 4)                   | [52]      |
| 4  | 6½  | M   | 103 | 0.14-0.07 gm/day                                     | 0.1 (fasting)<br>1.03 (post cibum)    | [49]      |
| 5  | 7   | M   | 86  | Positive   |                                       | [12]      |
| 6  |     |     | 100 | Like defective at high I Q<br><70                    |                                       | [5]       |
| 7  | 7½  | F   | 8   | FeCl <sub>3</sub> test stronger than in idiot sister |                                       | [54]      |
| 8  | 7½  | M   | 16  | 0.5 gm/day (secret)                                  |                                       | [39]      |
| 9  | 7½  | F   | 5   | Positive   |                                       | [ ]       |
| 10 | 8   | F   | 71  | 0.5-0.65 gm/day                                      | 2.3-2.9                               | [53]      |
| 11 | 8½  | F   | 0   | Positive   |                                       | [55]      |
| 12 | 11  | M   | 83  | 31 mg/100 ml   |                                       | [51]      |
| 13 | 13  | F   | 69  | 1.0 gm/gm creatinine                                 | 1.81                                  | [58]      |
| 14 | 29  | M   | 90  | Positive   |                                       | [58]      |
| 15 | 35  | M   | 89  | Positive   |                                       | [5]       |
| 16 | 50  | M   | 67  | 0.2-0.3 gm/gm creatinine<br>(sometimes negative)     | 1.33                                  | [50]      |

Mohr and Ruud [10] Case 3 of Armstrong and Tyler [8] and Case 21 of Wright and Tarjan [16] making a total of 20. The number is not greater than expected from the distribution of intelligence already discussed. The number of these cases helps to establish an incidence of

approximately 2 per cent with I Q above 60. This value can be used as a baseline to measure the effects of treatment on a group of phenylketonuric patients (see Treatment later in this chapter).

Several additional cases are described [9, 10, 59] who less certainly belong in the high grade group.

A compilation of these high grade phenylketonuric patients has disclosed that no serious neurologic defects occurred although the usual EEG abnormalities were found in several patients and one may have had seizures. Only in two was pigmentation more marked than expected. None could be described as normal. All had some degree of intellectual defect or emotional lability and minor neurologic changes such as hyperactive reflexes, rigid gait or tremors. The mild organic defects and abnormal behavior of these high grade patients reemphasize the evidences for a dissociated psychologic development described by Bickel and Grüter [52].

The metabolism of phenylalanine by these patients deserves to be studied. Metabolism through alternate pathways must be considered. In only 4 of the 16 reported patients was there evidence that the biochemical defect was milder than in the usual phenylketonuric patient. In 4 other well studied patients the biochemical defect was as severe as in typical patients. In the remaining 8 no evidence is available for estimating degrees of biochemical defect. Thus it is not established that a milder biochemical defect occurs in any of these patients.

There is no obvious genetic explanation for the occurrence of high grade phenylketonuric patients. Eight had severely affected phenylketonuric siblings. The son of another was severely affected. Only in one family was there a second relatively high grade patient. A variant (allelic) gene with a mild mental effect in these particular families is accordingly not an explanation. Having intellectually superior parents also fails to explain the difference in the degree of defect in sib pairs.

Heterozygosity is not a tenable explanation of the intelligence of some of these patients. The pedigrees within this group except for one father-son pair are typical of a recessive trait. There were no positive FeCl<sub>3</sub> tests on parents to indicate an incompletely recessive disease. The highest plasma phenylalanine concentration of a heterozygote on record is 0.20  $\mu$ moles per ml [62] which is well below the level of 0.9  $\mu$ moles per ml hypothesized as the threshold for excretion of significant amounts of phenylpyruvic acid [20, 50].

Environmental influences or modifying genes would appear to offer the most reasonable explanation for the high grade patients. Birth order seems to be a factor since at least 5 of the 8 patients with defective sibs were born after the defective sib. Birth order is unknown in the other 2 cases. In only 1 family was a defective child born after the high grade phenylketonuric child but even here another defective child was the first born [27].

It has been suggested that poor reabsorption of phenylalanine by the kidney tubules might lower the concentration of metabolites in the blood and tissues or alter the pattern of metabolism of the toxic metabolites. There is no evidence for such physiologic differences in the known high grade phenylketonuric patients. Altered carbohydrate metabolism has also been suggested. Of the 5 phenylketonuric sibs reported by Penrose [61] the 3 most intelligent were also diabetic or at least had glucosuria (IQ's as high as 55) while the two nonglucosuric phenylketonuric sibs were idiots.

It is to be expected that the degree of mental deficiency being a remote consequence of the reduced activity of an enzyme would vary much more than the degree of the enzyme defect. The existence of this group of high grade phenylketonuric patients does not support the view that the biochemical defect is causally unrelated to the mental defect nor does it suggest that the biochemical and mental defects are pleiotropic effects of the same gene. These cases do permit one to see the specific kind of intellectual damage done in its least complicated form. They also offer the encouragement that real improvement can be attained with rational therapy.

### CLINICAL FEATURES

Useful and independent clinical descriptions are provided by the reports of 50 cases by Jervis [21], 41 by Folling, Mohr and Ruud [10], 15 by Cowie [38], 21 by Wright and Tarjan [15] and 106 by Fauce [47]. The

TABLE 10-2 MAJOR CLINICAL FINDINGS IN PHENYLKETONURIA

| Finding                                  | Incidence per cent | Occurrence in low-grade patients |
|--|--------------------|----------------------------------|
| Agitated behavior                        | 90-92              | +                                |
| EEG abnormalities                        | 80                 | —†                               |
| Muscular hypotonicity                    | 75                 | —                                |
| Microcephaly                             | 68                 | +                                |
| Hypertensive reflexes                    | 66                 | +                                |
| Blond hair, blue eyes                    | 6                  | —                                |
| Inable to talk                           | 63                 | +                                |
| Hyperkinesia                             | 50                 | +                                |
| Unable to walk (and usually incontinent) | 35                 | +                                |
| Tremors                                  | 30                 | +                                |
| Eczema                                   | 13-34              | —                                |
| Seizures                                 | 6                  | +                                |

Percentages of incidences are approximately those reported in the larger series adjusted for redefinition of signs as given in Table 11-1. Finding occurring more frequently or severely in the low grade patient are marked +.

† The incidence of EEG abnormalities is not different in high and low-grade patient but the latter may have more obviously abnormal tracing.



author has used these as well as those in his own experience as the basis for evaluating smaller series or single cases. A tabulation of findings is given in Table 10-5.

The clinical descriptions of patients with phenylketonuria are still inadequate. A large number of rare characteristics have been described each by one or a few observers and sometimes denied by other authors. These include kyphosis, pes planus, spina bifida, syndactylia of toes, intraventricular heart block, hypogonitism, dermatographia, light sensitivity, hypersegmentation of neutrophil blood cells, decreased galactose tolerance, and slightly increased basal metabolism. Acrocyanosis or poor peripheral circulation, is frequently mentioned but is probably not peculiar to the disease [62]. Many phenylketonuric patients have an indisputable smell variously described as a "mousy," "mousey," "wolf-like" or "barny" odor. It is referable to the phenylacetic acid excreted by these patients. This smell may account for the "hyperhidrosis" often mentioned. The allegation that these patients excrete phenylacetic acid in their sweat has not been demonstrated chemically.

### *Development*

The usual developmental landmarks—the times of sitting, walking, and talking—are sometimes reached at normal ages but usually are delayed. At the latest ages at which the performance would normally be expected, 35 per cent could not walk and 63 per cent could not talk. Sphincter control is absent in most of those who cannot walk. Dentition is often delayed until after the eleventh month. Blumey and Gulliford [65] found enamel hypoplasia in 3 patients. No other developmental abnormalities have been regularly observed.

### *Appearance*

**Body Size, Weight, and Shape.** Children with phenylketonuria are often under standard height and weight for ages and the adults smaller than average [47]. Large or overweight individuals are seldom seen. It must be remembered that adequate food intake is difficult to achieve in very retarded individuals. The general physical development is at least as good as that of nonphenylketonuric individuals of comparable intellectual level. Microcephaly is a regular finding in over half the cases [47]. It is more marked in the low grade patients. Head circumference averages nearly 2 cm smaller than normal [63]. Prominent or prognathic maxillae with consequent widening of the interdental spaces have been described as a typical configuration of the skull [16, 51, 64].

**Stance and Gait.** A stiff gait with short steps, a 'stooping walk,' or a stumbling gait is often described. A pathicoid stance is sometimes seen and in severely defective patients a tailorlike sitting position or Schnobidersitz [54] is typical (Fig. 10-3). The abnormalities of

muscle tone contributing to these changes are described with the neurologic signs.

**Skin Lesions** Eczema usually beginning in infancy occurred in 19 per cent of Paine's cases. In the majority it persisted to adolescence or adulthood. The incidence was 34 per cent in the author's survey of 104 published cases. In one-third the eczema was said to be severe and one



Fig. 10-3. The characteristic *Schöder st* of severely defective phenylketonuric patient. (By permission of K. Laeg [13].) Incessant rhythmic motions complete the usual picture [5].

patient died with generalized eczema. A dry or rough skin was often noted. Some of these skin manifestations can be attributed to unhygienic conditions and to a light and sensitive skin. Fölling et al [10] emphasized the frequency of the same affections in normal individuals. The one published dermatologic study [32a] demonstrated that phenylketonuric skin was normally tanned by the sun and was not abnormally sensitive to sunlight.

**Pigmentation** Deficient pigmentation with blond hair and blue eyes is characteristic of phenylketonuria. The coloration is not abnormal in

type and was not noted in Folling's original description from Oslo where most individuals have a similar coloration. In Paine's cases from the northeastern United States 64 per cent had blue eyes and 17 per cent brown eyes. Sixty per cent had blond hair and the remainder light brown or brown. Berk and Stern [64] demonstrated a significantly lighter iris color in 26 phenylketonuric mentally defective patients than in controls and lighter irides in 12 phenylketonuric patients than in 23 of their unaffected siblings. There was no relation to IQ. Even though the incidence is higher among Nordic people each patient has a relatively lighter complexion than other members of his family. The dilution of hair color in phenylketonuric patients compared with their unaffected relatives has been demonstrated by reflecting spectrophotometry [38]. Striking instances of blond phenylketonuric patients in darkly pigmented families of Sicilian [3] or Spanish [22] origin have been reported. Another striking case was said to be a mulatto [20]. He had sandy blond hair and blue eyes but negroid fundi. The physical characteristics of the one other Negro patient were not described [23]. Phenylketonuric Japanese patients have brown hair [26-27]. The normally pigmented iris of the brown such as the substantia nigra and locus ceruleus may lack pigmentation.

### *Nervous System*

The vast majority of patients show a series of typical neurologic changes running parallel to the degree of mental defect and in general worse in severely defective individuals. Traces of the same abnormalities are evident in the high grade patients.

**Epilepsy** A history of convulsive seizures appears in 26 per cent of Paine's series plus the 418 cases reported by Jervell [12]. Seizures usually began during the first 18 months of life and stopped spontaneously before adulthood. The incidence of seizures was higher and the attacks were more severe in the severely defective patients. Recurrent episodes of staring or inattentiveness after 6 months of age are very frequently recorded. The incidence of the *petit mal* attacks plus overt convulsions gives an overall incidence of abnormal cerebral activity which is probably over 50 per cent. The epilepsy is amenable to the usual therapy. Prolonged severe epilepsy is exceptional but it has been noted as a complication of the low phenylalanine diet therapy.

**Electroencephalogram** The majority of phenylketonuric patients have abnormal EEG patterns regardless of whether or not they have had seizures. The incidence is approximately the same with low or high grade defective patients. Paine [41] found 78 per cent abnormal recordings in 33 cases (75 per cent were abnormal in 24 of these cases who never had seizures). For Rosenberg and Gibbs [66] reported 93 per cent abnormal tracings in 19 patients. Their investigation included the technique of sleep recording by means of which disorders of this type are best seen.

The abnormalities consist of spike and wave complexes and the petit mal variant type (even in some individuals without epilepsy). The most common finding is a mixture of high voltage fast and slow waves occurring more irregularly than in petit mal. Fors et al likened the disorganized sleep patterns to those seen in hydrocephalus with injury to the thalamus or hypothalamus. These abnormalities are not pathognomonic but they do suggest deep mid line brain damage.

**Behavior** The statement of Wright and Tarjan [15] is most apt. None could be described as friendly, placid or happy. They are restless, jerky and fearful individuals. Their behavior ranges from that of the highly anxious and restless high grade patient to the destructive and noisy psychotic episodes observed in 10 per cent of the patients. Night terrors beset the higher grade patients. Uncontrollable temper tantrums are common (32 per cent in Laine's series). The hyperactivity, irritability and uncontrollable temper are the usual reasons given for admitting these patients to institutions.

**Muscular Hypertonicity** Jervis found increased muscle tone in 70 per cent and Wright and Tarjan in 76 per cent. The patients seem always ready to jump. This unrelaxed attitude may be responsible for the awkward or even rigid, short stepped gait with few associative movements that is seen in 20 per cent of the low grade group. Approximately 20 per cent are described as having hypotonic muscle tone. Jervis also described cog wheel rigidity. This finding is particularly difficult to evaluate in the patients.

**Hyperactive Tendon Reflexes** Two thirds of all patients show abnormally brisk tendon reflexes and in a sizable fraction ankle and patellar clonus can be elicited. Clonus persists continuously in some patients.

**Adventitious Hyperkinesia** A great variety of abnormal body movements has been described. Each patient has his own favored motions out of the infinite variety possible. The important point is his restless movement. There are voluntary, purposeful and repetitive motions of the whole or a part of the body. There may be inconspicuous fiddling movements of the fingers or violent body swinging which continues for hours. The persistent ankle clonus may be of this type. Each patient has a limited repertoire of these movements but if one motion is suppressed others occur. They contribute a great deal to the picture of behavioral unrest and agitation. Because of their combination with muscular rigidity they are often described as choreiform or athetoid.

**Tremors** A fine rapid and irregular tremor of the outstretched hands was seen in 30 per cent of Laine's series. It may become stronger on volition and may spread to other parts of the body. Together with increased tendon reflexes and jerky rigidity it is one of the reasons for suspecting extrapyramidal disease in these patients even though true spasticity may not be present.

**Other Neurologic Findings** A small percentage of phenylketonuric patients has signs of severe brain disease in association with severe mental defect. These are the patients described by Jervis [21] as the 4 per cent with "stationary severe cerebral palsy with diplegia, contractures and pyramidal signs" and by Paine [47] as 5 per cent with spastic para- or tetraplegia. There are rare reports of positive Babinski or Hoffman signs [27, 38]. The relationship of these findings to phenylketonuria is not clear. The patients may have other concurrent diseases or the signs may represent the ultimate degree of cerebral defect caused by phenylketonuria.

**Pneumoencephalographs** Pneumoencephalographs of patients have shown evidence of diffuse cortical atrophy [21-67].

### *Pathologic Findings*

No relevant pathologic changes occur outside the central nervous system in phenylketonuria. The changes in the central nervous system which have been observed do not account for the mental deficiency. Occasional reports have emphasized nonspecific *hinter abnormalities* or such changes as hypoplasia of the pituitary or gonads. Table 10-2 lists the autopsies that have been performed. Most of the deaths occurred in young individuals after long and debilitating illness that might produce a variety of irrelevant pathologic changes. One frequent finding is that the weight of the brains is about two-thirds of normal.

The first autopsy reported on a phenylketonuric subject disclosed another rare disease, neurofibromatosis, with peripheral nerve tumors but without cutaneous nodules or pigmentation. This incidental finding delayed the recognition that phenylketonuria is an extremely subtle and unsolved problem in neuropathology. The second recorded autopsy was little more helpful since it was performed on an exhumed body [35].

The complete list of autopsies presented here is more extensive than any previously evaluated. Many of the findings can be attributed to coincidental abnormalities to the effect of the final illness, or to artifacts. Peters [68] concluded his discussion of the subject: *Aus den wenigen anatomisch pathologischen Befunden lässt sich ein charakteristisches pathomorphologisches Syndrom nicht erkennen. Auch über Pathogenese und Ursache lässt sich an Hand der centralnervösen Veränderungen kein Schluss ziehen.*

The listing of cases by age (Table 10-2) reveals unexpected support for one vaguely defined pathologic process. An abnormality of myelination in phenylketonuria of the sort first suggested by Alford et al [32] remains a possibility. This view was extended by Scholz [33]. It is based on the findings in the youngest patients (under 5 years of age). It could account for the negative findings in many of the patients who were past the age at which the abnormalities could be clearly seen without detailed

cytopathologic studies Scholz hypothesized that there is a slowing and possibly an inhibition of myelination. At the same time glial cells especially those concerned with myelination develop normally or excessively. This picture is seen only in the youngest patients. The ultimate result in older patients would be an intensive gliosis of oligodendrocytes and astrocytes with more or less normal appearing myelin. Pathologic evidence for the deficient myelination is available in all the youngest patients studied and in some of the older patients there is evidence of gliosis although often with an apparently normal degree of myelination. The best studied cases are the ones which support this picture of the disease process. Myelination normally occurs during the first few years of life during the period when the intellectual defect in phenylketonuria is established.

Deficient myelination is well described in younger subjects. The youngest child examined was 1½ years of age [37]. The brain showed a marked lack of myelination of the optic cortical cerebellar and spinal tracts. The overall extent of myelination was between that found in an 8 month-old fetus and a 2 month-old child. The next oldest died at 1½ years in an acute crisis [68]. She had been treated with a low phenylalanine diet for 5 months (see Treatment further on). There was a lack of myelination in the optic nerve and tract and in other regions of the brain and spinal cord. Myelination was equivalent to that of a 5-month-old infant. There were excessive oligodendrocytes and astrocytes which increased the consistency of the brain. Paradoxically the gliosis increased in a caudal direction. A 3 year-old child was stated only to have some paleness of myelin in the temporal regions [18]. Another was 5½ years old [59]. The brain also showed a patchy deficiency of myelin which was less marked than in the younger patient observed by the same authors. A 3½ year-old not included in Table 10-2 died in hypoglycemia after chronic malnutrition during 2 months treatment with a low phenylalanine diet. The only neuropathologic changes were acute and consistent with hypoglycemic encephalopathy [69].

Older children and adults may or may not show findings consistent with the younger patients. The paleness of myelin and gliosis in the 15-year-old patient of Leon [37] is suggestive. The 33 year-old patient of Alvord et al [5] had myelin loss in the center of the optic chiasma and an intense honeycomb-like gliosis surrounding the empty spaces. The patient of Sander [44] had a sieve-like process in the extrapyramidal centers. The 2½-year-old patient of Corsellis [79] showed an intense gliosis of the occipital lobes but only a few small spots of demyelination perhaps referable to epilepsy early in life. The uncommonly dense cytoarchitecture in the cortex of Larson's [39] 20-year-old patient should also be mentioned. It is possible that additional cases showing destruction of white matter could be fitted into the still hypothetical pathologic process.

Chemical analysis of these brains might provide valuable insight into the cause of defective myelination. This process which involves the elaboration of several types of complex lipids and their lamination in lipoprotein layers about the nerve fiber is complicated [81]. Myelin is not a simple chemical substance but a complex deposition of lipid, largely

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### *Pathologic Findings*

No relevant pathologic changes occur outside the central nervous system in phenylketonuria. The changes in the central nervous system which have been observed do not account for the mental deficiency. Occasional reports have emphasized non-specific liver abnormalities or such changes as hypoplasia of the pituitary or gonads. Table 10-2 lists the autopsies that have been performed. Most of the deaths occurred in young individuals after long and debilitating illness that might produce a variety of irrelevant pathologic changes. One frequent finding is that the weight of the brains is about two-thirds of normal.

The first autopsy reported on a phenylketonuric subject disclosed another rare disease—neurofibromatosis with peripheral nerve tumors but without cutaneous nodules or pigmentation. This incidental finding delayed the recognition that phenylketonuria is an extremely subtle and unsolved problem in neuropathology. The second recorded autopsy was little more helpful since it was performed on an exhumed body [32].

The complete list of autopsies presented here is more extensive than any previously evaluated. Many of the findings can be attributed to coincidental abnormalities to the effect of the final illness or to artifacts. Peters [68] concluded his discussion of the subject: *Aus den wenigen anatomisch pathologischen Befunden lässt sich ein charakteristisches pathomorphologisches Syndrom nicht erkennen. Auch über Pathogenese und Ursache lässt sich an Hand der zentralnervösen Veränderungen kein Schluss ziehen.*

The listing of cases by age (Table 10-2) reveals unexpected support for one vaguely defined pathologic process. An abnormality of myelination in phenylketonuria of the sort first suggested by Alvord et al [32] remains a possibility. This view was extended by Scholz [33]. It is based on the findings in the youngest patients (under 5 years of age). It could account for the negative findings in many of the patients who were past the age at which the abnormalities could be clearly seen without detailed

porated into the body protein of the different cells. Phenylalanine is also oxidized in the liver to tyrosine. The reverse reaction does not occur.

Tyrosine undergoes further reactions in various specialized tissues. The largest dissimilative reaction of tyrosine occurs in the liver; there is a small reaction in the kidney. This is oxidative ring fission to components of the citric acid cycle. In this way tyrosine is eliminated as an aromatic compound. The amount of tyrosine handled in other ways is

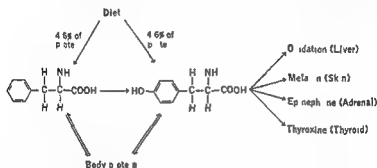


Fig 10-4 Diagram of normal metabolic flow of phenylalanine and tyrosine and the location of the reaction blocked in phenylketonuria ( )

very much smaller though no exact quantitative comparisons can be made. These smaller amounts of tyrosine are metabolized in specialized organs such as the skin, adrenal glands, and thyroid.

### Phenylalanine Metabolism

The unnatural form of phenylalanine, like most other  $\alpha$ -amino acids, is oxidatively deaminated by  $\alpha$ -amino acid oxidase. This reaction forms phenylpyruvic acid, which is identical with the typical metabolite of phenylketonuria. Since the further metabolism of this compound is rate limited, the administration of modest amounts of DL-phenylalanine is followed by the appearance of phenylpyruvic acid in the urine. This has been known since the early work of Kotake, Masai, and Mori [73]; it was confirmed by Chandler and Lewis [74] and on it was based one of the first theories of the nature of phenylketonuria. Instead of absence of a normal enzyme of metabolism, this theory suggested an abnormal enzyme (a racemase) which converted L- to D-phenylalanine, which in turn gave rise to the phenylpyruvic acid [10]. However, Fölling found only L-phenylalanine in blood and urine in phenylketonuria, and this was confirmed by the microbiologic assays of Prescott et al [7a]. This route of phenylpyruvic acid formation may confuse experiments when DL-phenylalanine is used, but it is otherwise unrelated to the reactions primarily involved here.



sphingomyelin and phosphatidylethanolamine in addition to proteolipids, cholesterol and protein [70]. It is premature to suggest a direct connection between phenylalanine and tyrosine metabolism and this complex chemical event, but interference with this process by the abnormal chemical milieu of the phenylketonuric brain is quite possible.

**Associated Conditions** Phenylketonuria has been associated with other rare conditions, but usually only in single cases. Some of these may be other recessive conditions that show up in the same matings which permit phenylketonuria. It has been associated with Cowder's muscular dystrophy, neurofibromatosis, and Schilder's disease. To these can be added the association with cri-du-chat [71] and with a second metabolic defect characterized by  $\alpha$ -hydroxybutyric acid excretion [72]. There is no indication that such associations are other than coincidental.

## BIOCHEMICAL ABNORMALITIES

The normal oxidation of L-phenylalanine to tyrosine in the liver is almost completely stopped in phenylketonuria. The evidence is quite complete that hereditary inactivity of the enzyme for this reaction, phenylalanine hydroxylase, is the primary phenotypic lesion in this disease. The failure to convert the essential amino acid, phenylalanine, to tyrosine causes directly and indirectly a host of biochemical, physiologic, and pathologic repercussions.

Not only is there accumulation of the phenylalanine which is constantly fed into the organism by the diet and which must seek the other avenues of removal (and a relative deficiency of tyrosine, which becomes an essential dietary amino acid), but a variety of intracellular systems are called upon to adjust their function to this new metabolic state. The renal tubular transport system, which normally reabsorbs relatively small amounts of phenylalanine, is faced with the reabsorption of amount approaching its maximum transfer capacity. Amino acids that are normally concentrated in cells for purposes of growth and metabolism must continue to be taken up in appropriate amounts despite the high concentration of phenylalanine seeking entry. Each amino acid residue must continue to be incorporated accurately into the myriad proteins synthesized by the cells regardless of the distorted pattern of free amino acids in the intracellular pool. It is remarkable indeed that so few of the functions of the body are seriously impaired in phenylketonuria. The relative lack of damage indicates that all processes but one are intact and that biologic processes have great adaptability for meeting new conditions.

## PRELIMINARY CONSIDERATIONS

The metabolic flow of phenylalanine and tyrosine appears in Fig. 10-4. The amino acids are continuously supplied from the diet and are incor-

### The Common Pathway of Amino Acid Metabolism

Nearly every amino acid has a highly individual pathway by which it is metabolized. Since most amino acids also participate in oxidative deaminations by the L- or D-amino acid oxidases and in transaminations to the  $\alpha$ -keto acids, there is also potentially a common pathway of degradation through the keto acids to the next lower carboxylic acids as shown in Fig. 10-5. The major metabolites found in phenylketonuria

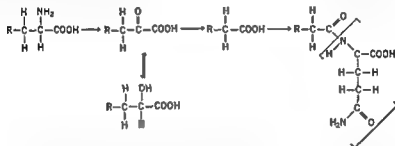


Fig. 10-5 A common non-specific pathway of amino acid degradation through keto and lactic acids, acetic acid, and conjugates of the latter. Such secondary pathways account for many of the metabolites in phenylketonuria.

find representation in such sequences of reactions. The parent amino acids are phenylalanine, tyrosine (or its  $\alpha$ -keto derivative), tryptophan, and the hypothetical compound  $\alpha$ -tyrosine. The classification of the metabolites in phenylketonuria according to a hypothetical series of reactions is illustrated in Table 10-6. Some of the intermediates expected according to this scheme have not been found. The degradation of  $\alpha$ -tyrosine may occur by another route (through  $\alpha$ -tyramine) for which enzymes are known to exist in the body. Since this scheme was first presented [14], one of the predicted metabolites, indolepyruvic acid, has been added to the list known to be formed in phenylketonuria [76].

### BIOCHEMICAL DIAGNOSTIC CRITERIA AND METHODS

The vast majority of phenylketonuric patients have been recognized solely by their excretion of phenylpyruvic acid as identified by the simple  $\text{FeCl}_3$  test. This single item of qualitative information has been extraordinarily successful in distinguishing phenylketonuria. Diagnosis of crucial cases should be supported by additional chemical criteria. The most valuable of these are increased plasma phenylalanine concentration and the excretion of  $\alpha$ -hydroxyphenylacetic acid.

**The  $\text{FeCl}_3$  test.** The addition of several drops of 5 per cent  $\text{FeCl}_3$  to 5 ml fresh urine first produces some precipitation of phosphates; then as more is added to positive specimen, there appears after 2 or 3 min. an olive-

TABLE 10-6 THE UNUSUAL METABOLITES IN I HENYLETONOLIA

Parent amino acid Phenylalanine

| Parent amino acid | Phenylalanine |  |  |  | o-Tyrosine          |  |  |  | Tyrosine            |  |  |  | Tryptophan  |  |  |  |
|-------------------|---------------|--|--|--|---------------------|--|--|--|---------------------|--|--|--|-------------|--|--|--|
|                   | Phenyl = R    |  |  |  | o Hydroxyphenyl = R |  |  |  | p Hydroxyphenyl = R |  |  |  | Indolyl = R |  |  |  |
| Derivatives       |               |  |  |  |                     |  |  |  |                     |  |  |  |             |  |  |  |
| pyruvic           |               |  |  |  |                     |  |  |  |                     |  |  |  |             |  |  |  |
| lactic            |               |  |  |  |                     |  |  |  |                     |  |  |  |             |  |  |  |
| acetic            |               |  |  |  |                     |  |  |  |                     |  |  |  |             |  |  |  |
| acetyl conjugate  |               |  |  |  |                     |  |  |  |                     |  |  |  |             |  |  |  |

Arranged as products of parent amino acid reaction with 10-5 C in per cent with also in parentheses

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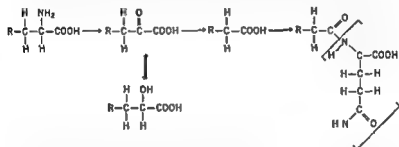


Fig. 10-5 A common nonspecific pathway of amino acid degradation through keto and lactic acids, acetic acids, and conjugates of the latter. Such secondary pathways account for many of the metabolites in phenylketonuria.

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**The  $\text{FeCl}_3$  test.** The addition of several drops of 5 per cent  $\text{FeCl}_3$  to 5 ml fresh urine first produces some precipitation of phosphates; then as more is added to positive specimens, there appears, after 2 or 3 min, an olive-

green color which fades slowly over the next hour or two. Acidification is not necessary.

The test is sometimes negative in phenylketonuria when the urinary concentration of phenylpyruvic acid is less than 0.2 mg per ml. This occurs in very dilute urine in rare individuals with low phenylpyruvic

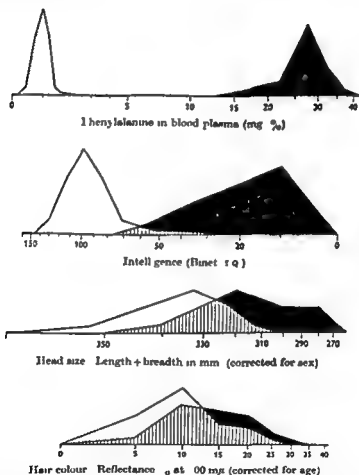


Fig 10-6 Frequency distributions of some characteristic features of phenylketonuria in phenylketonuric patients (right) and in control populations (left) (By permission of H. Harris [63])

acid output with low protein intake or in day old urine after the relatively labile phenylpyruvic acid has decomposed. Gibbs and Woolf [30a] give 0.05 mg per ml as the limit of detection with ferric chloride. The color results from the chelation of  $\text{Fe}^{3+}$  with the enol group of the phenylpyruvic acid. The analogous reaction of  $\text{Fe}^{2+}$  with acetone is used

is a standard chemical test. Acetone, acetoacetic acid or salicylic acid gives distinctive colors in this test that are not confused with the typical green reaction of phenylpyruvic acid. A green color similar to that given by phenylpyruvic acid occurs with a metabolite of chlorpromazine [77] and at times in urines containing bile. Ephemeral green colors caused by *p*-hydroxyphenylpyruvate were found in urines of 7 out of 1141 infants screened by Cribbs and Woolf [30a]. Confirmation of the presence of phenylpyruvic acid is obtained by extracting it from acid solution with ether and repeating the  $\text{FeCl}_3$  test [78] or by forming the insoluble 2,4-dinitrophenylhydrazone [16]. Both tests can be used for quantitative determinations. Conditions for increasing the sensitivity of the ferric chloride test were described by Safer and Harris [78a].

References for the determination of plasma phenylalanine and urinary *o*-hydroxyphenylacetic acid will be found in the next section.

The need for complete reliance upon chemical criteria for the diagnosis of phenylketonuria can be seen from the frequency distribution of certain characteristics of phenylketonuria measured in normal and phenylketonuria populations (Fig. 10-6). Only the biochemical criterion furnishes absolute discrimination between normal and phenylketonuric persons even though the two populations differ significantly with respect to certain of the overlapping characteristics.

#### CHARACTERISTIC METABOLITES

There are no abnormal metabolites in phenylketonuria; only normal metabolites in abnormal amounts. Fölling [1] isolated and identified phenylpyruvic acid as part of the original description of the disease. Later [79] he found elevated amounts of *L*-phenylalanine and probably phenyllactic acid in urine and *L*-phenylalanine in serum. The true significance of the elevated plasma phenylalanine indicating a block in the metabolism of the phenylalanine itself became clear with the confirmation of the high phenylalanine levels by Jervis. Block, Bolling, and Kanze [4]. Jervis [5] then demonstrated the block by showing that no rise in blood tyrosine level occurs in the plasma of phenylketonuric patients after a dose of phenylalanine. It then became clear that phenylalanine is the primary metabolite which accumulates and that the other metabolites are derivatives of it. Later other compounds (derivative of tyrosine and tryptophan) were recognized as arising secondarily from separate metabolic reactions. The compound now known to be present in abnormal amounts are described below and listed in Table 10-7, 10-8.

**Phenylalanine.** Probably the most accurate plasma phenylalanine concentrations are those determined by column chromatography which gives the lowest values of the several methods. Slightly higher values are obtained with the specific enzyme decarboxylase method and still

green color which fades slowly over the next hour or two. Acidification is not necessary.

The test is sometimes negative in phenylketonuria when the urinary concentration of phenylpyruvic acid is less than 0.2 mg per ml. This occurs in very dilute urines in rare individuals with low phenylpyruvic

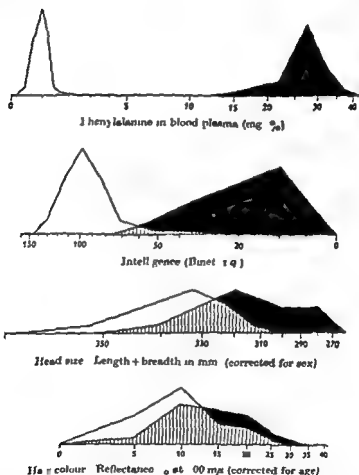


Fig. 10-6. Frequency distribution of some characteristic features of phenylketonuria in phenylketonuric patients (right) and in control populations (left). (By permission of H. Harris [63].)

acid output with low protein intake or in dry old urine after the relatively labile phenylpyruvic acid has decomposed. Cebbs and Woolf [30a] give 0.02 mg per ml as the limit of detection with ferric chloride. The color results from the chelation of  $\text{Fe}^{3+}$  with the enol group of the phenylpyruvic acid. The analogous reaction of  $\text{Fe}^{3+}$  with acetone is used

*o*-tyramine The last a probable precursor of *o*-hydroxyphenylacetic acid [97] is formed from  $\pi$  tyrosine and concentrated in brain and to a lesser extent in liver

### *Quantities of Urinary Metabolites*

Many of the values recorded in the literature for the metabolites excreted in abnormal amounts in phenylketonuria are not useful. There is great difficulty in collecting all urine for a given period from these patients. There is also uncertainty about body size and protein intake of the patients in many reports. In Table 10-8 only the urinary excretion values have been included which give a basis for comparison such as the urinary nitrogen or creatinine.

**Phenylpyruvic Acid** The phenylpyruvic acid excretions recorded by Jervis [88] have been expressed per 15 gm urinary nitrogen to approximate the daily output of an adult. The remarkable constancy within this group which included children as well as adults shows that phenylpyruvic acid excretion in these patients is largely a function of protein intake. The larger variation seen in the series of Armstrong and Low [20] which is expressed as per grams creatinine may be the result of varying protein intakes. Creatinine excretion reflects body size but not intake although the two are roughly parallel under usual circumstances. One patient who excreted only 0.3 gm phenylpyruvic acid per gram creatinine and who was a high grade defective (I Q 67), has been omitted from the second series.

**Phenyllactic Acid** Zeller [98] isolated and identified phenyllactic acid from phenylketonuric urine. Few measurements have been reported but it is excreted in quantities intermediate between those of phenylpyruvic acid and phenylalanine.

**Phenylalanine** Normally phenylalanine is excreted in amounts of 10 to 15 mg per gm creatinine as determined by column chromatography [99] although children under 1 year excrete several times as much of this. It can be a sensitive indicator of the blood level if careful measurements are made.

***o*-Hydroxyphenylacetic Acid** Armstrong, Shaw and Robinson [91] discovered this unexpected metabolite of phenylalanine in phenylketonuric urine. Amounts less than 1 mg per day are normally excreted. The excretion of a high grade patient (Case 16 Table 10-4) was the lowest in the series of measurements used for the table (0.08 gm per gm) and has been omitted.

**Indolepyruvic, Indolelactic and Indoleacetic Acids** Indolelactic and indoleacetic acids were discovered in abnormal amounts in phenylketonuric urine by Armstrong and Robinson [92] and confirmed by Jepson [100]. Schroter and Flug [77] completed this group with the discovery of the very labile pyruvic acid derivative. These arise from tryptophan.



higher ones with the microbiologic and paper chromatographic method. Accepted normal values are under  $0.1 \mu\text{mole per ml}$  ( $1.65 \text{ mg per } 100 \text{ ml}$ ). Much higher concentrations in normal persons are given by the Kapeller Adler chemical method ( $0.18$  to  $0.111 \mu\text{moles per ml}$  or  $3$  to  $5 \text{ mg per } 100 \text{ ml}$ ) which has frequently been used to measure the high levels found in phenylketonuric patients [59]. It is not recommended for the lower normal values [91, 92].

Plasma phenylalanine concentrations in heterozygotes of phenylketonuria are definitely higher than those in normal persons as determined by the same method but the difference is small and demonstrable only with the most precise methods of analysis.

The markedly elevated plasma phenylalanine in phenylketonuria has been measured by column chromatography in only one patient but this agreed well with the means of the series measured microbiologically and with the enzyme method. Several series of determinations in phenylketonuria by the Kapeller Adler method are included in the table. This method though admittedly inaccurate in this range [59] is said to give internally consistent results. With this method higher phenylalanine values were found in phenylketonuric children under 2 years of age as is shown in the table. Other methods have not shown such striking differences in young children.

The phenylalanine level in cerebrospinal fluid is approximately one-fourth that in the plasma and the level in sweat is still lower.

**Phenylpyruvic Acid.** Jervis [89] was able to measure phenylpyruvic acid in the plasma of phenylketonuric patients but he was unable to detect it in normal plasma. The low concentration in plasma is referable to renal excretion which may occur in part by active tubular secretion. The evidence for this is that probenecid, an inhibitor of renal transport, decreased the renal excretion of phenylpyruvic acid by one-half and simultaneously increased the blood level of this compound [89]. The compound may also be actively secreted by the skin since the concentration of phenylpyruvic acid in sweat is substantially higher than in plasma.

**Other Compounds in Blood.** **Phenylacetic Acid, 5-Hydroxytryptamine, Epinephrine.** Jervis [12] mentioned that phenylacetic acid is present in the blood of phenylketonuric patients but the amount must be very low. Most of the other metabolites formed in abnormal amounts by phenylketonuric patients have been detected only in urine. Mention should be made of the pharmacologically active substances which occur in concentrations an order of magnitude lower than those described. These include 5-hydroxytryptamine which is lower than normal in the plasma in phenylketonuria (inferred from measurements on serum from incubated clotted blood) [90], epinephrine which is lower than normal in the plasma of all mental defectives [90] and the still hypothetical intermediate

of phenylacetylglutamine. Stem Paladini, Hirs and Moore [102] reported that 250 to 500 mg per day are excreted by normal individuals and that this accounts for one half of the conjugated glutamic acid in urine. They examined by column chromatography one patient with phenylketonuria who excreted 2.4 gm per day. Excretions between 300 and 450 mg per day can be calculated from data on two phenylketonuric children studied by Meister, Udenfriend and Bessman [103]. Coates et al [49] reported figures of 385 to 640 mg per day in their patient with a relatively high I.Q.

TABLE 10-8 AMOUNTS OF URINARY METABOLITES IN PHENYLKETONURIA

| Compound                     | N  | Mean             | Range   | Ref.     |
|------------------------------|----|------------------|---|----------|
| Phenylpyruvic acid           | 20 | 2.13             | 1.45-7.72 gm/15 gm N                          | [59]     |
|                              | 27 | 2.27             | 0.8-5.6 gm/gm creat                           | [10]     |
| Phenyl lactic acid           | 20 | 0.93             | 0.60-1.35 gm/15 gm N                          | [59]     |
| Phenyl glyoxylic acid        | 20 | 0.45             | 0.30-0.75 gm/15 gm N                          | [59]     |
| o-Hydroxyphenyl lactic acid  | 12 | 0.22             | 0.10-0.40 gm/gm creat                         | [51]     |
| Indole pyruvic acid          | 2  |                  | 0.11-0.17 gm/d y (mean)<br>s.e. = 0.04 gm/d y | [70]     |
| Indole lactic acid           | 15 |                  | 0.01-0.15 g/gm creat                          | [52]     |
| Indole lactic acid           |    |                  | Am. t. t.                                     |          |
| o-Hydroxyphenyl lactic acid  | 9  | 4.9 ± 0.7 (S.E.) | 1.6-8.6 mg/gm creat                           | [50]     |
| Phenyl lactic acid           | 9  | 2.9 ± 0.2 (S.E.) | 1.6-3.6 mg/gm creat                           | [50]     |
| Phenyl pyruvic acid          | 8  |                  | 0.03-2.4 gm/d y                               | See text |
| Phenyl lactic acid           |    |                  | Am. t. t.                                     | [55]     |
| o-Hydroxyphenyl pyruvic acid |    |                  | T. a. s. reported                             | [55]     |

Unpublished determinations by column chromatography done in the author's laboratory by C. D. Thron give 760 mg per gm creatinine for the high grade phenylketonuric patient described by Hsia et al [58] and 690 mg per gm creatinine for the severely retarded patient studied in comparison.

**p-Hydroxyphenylpyruvic, -lactic, and -acetic Acids** These metabolites similarly to the indole acid arise secondarily from tyrosine metabolism. The compounds may be detected by paper chromatography and the amounts excreted are not known.

#### Dynamic Interrelationships of Metabolites

Certain correlations have been noted among the quantities of the various metabolites in phenylketonuria. The dietary treatment of phenylketonuria has made it clear that the plasma level of phenylalanine reflects the amount in the diet. So does the amount of phenylpyruvic acid excreted as already deduced from its constancy in relation to urinary nitrogen. Consequently there is a rough correlation of the serum phenyl

tophan metabolism. Indoleacetic acid is said to be excreted in larger amounts than indolelactic acid, but no figures are available [92]

**5-Hydroxyindoleacetic Acid** 5 Hydroxyindoleacetic acid excretion was first found to be slightly low in phenylketonuria by Armstrong and Robin on [92]. This was confirmed by Ferrari, Campagnari and Guida

TABLE 10-7 HIGH CONCENTRATIONS OF METABOLITES

| Metabolite                  | N / patient | Mean $\pm$ SD $\mu$ mol / mol | Range $\mu$ mol / mol | Method                | Reference |
|-----------------------------|-------------|-------------------------------|-----------------------|-----------------------|-----------|
| Phenylalanine               |             |                               |                       |                       |           |
| Urine                       | 14          | 0.051                         | 0.040-0.064           | Column chromatography | [41]      |
| Plasma                      | 106         | 0.078 $\pm$ 0.013             | 0.040-0.115           | 2 yme                 | [77]      |
| Urine                       | 17          | 0.044 $\pm$ 0.003 (9 F)       | 0.034-0.079           | Urology               | [4]       |
| Plasma                      | 17          | 0.094 $\pm$ 0.019             | 0.047-0.260           | Urology               | [45]      |
| Urine                       | 10          | 0.10 $\pm$ 0.04               | 0.01-0.151            | Paper chromatography  | [45]      |
| Indolepropionic acid        |             |                               |                       |                       |           |
| Urine                       | 2           | 0.103 $\pm$ 0.016             | 0.075-0.205           | 2 yme                 | [64]      |
| Plasma                      | 10          | 0.151 $\pm$ 0.054             | 0.045-0.234           | Paper chromatography  | [45]      |
| Phenylalanine               |             |                               |                       |                       |           |
| Plasma ( )                  | 1           | 1.42                          |                       | Column chromatography | [42]      |
| Urine                       | 18          | 1.88                          | 1.15-2.1              | Urology               | [6]       |
| Plasma (serum)              | 34          | 1.78                          | 1.03-3.1              | Urology               | [51]      |
| Plasma (serum)              | 12 (<2 y)   | 4.05                          | 16-6.00               | Urology               | [40]      |
| Plasma (serum)              | 4 (>2 y)    | 4.8                           | 1.33-3.21             | Urology               | [1]       |
| Plasma (serum)              | 18 (11 gro) | 5.4                           | 1.31-3.82             | Urology               | [41]      |
| Urine                       | 9           | 0.44                          | 0.37-0.50             | Urology               | [41]      |
| Urine                       | 10          | 0.70                          | 0.043-0.104           | Urology               | [41]      |
| 5-Hydroxytryptamine         |             |                               |                       |                       |           |
| Urine                       | 39          | 0.043 $\pm$ 0.015             | 0.019-0.104           | Urology               | [40]      |
| Plasma                      | 10          | 1.55                          | 0.34-3.40             | Urology               | [41]      |
| 5-Hydroxytryptamine (serum) |             |                               |                       |                       |           |
| Urine                       | 9           | 270 $\pm$ 51 (9 F) (mg / ml)  | 77-808                | Urology               | [90]      |
| Phenylalanine               | 9           | 57 $\pm$ 11 (9 F) ( )         | 11-10                 | Urology               | [90]      |

[101]. The measurements shown in the table by Pare et al [90] accompanied the demonstration of low serum 5 hydroxytryptamine (serotonin) cited in Table 10-7.

**Phenylacetylglutamine and Phenylacetic Acid** Woolf [93] detected phenylacetic acid for which no satisfactory methods of measurement are available and also its principal conjugation product with glutamine. Small amounts are said to be conjugated with glucuronic acid [12].

No definite quantitative statements can be made about the excretion

of the usual symptoms of untreated phenylketonuria [8-53] Woolf et al [54] described day to day variations in behavior correlated with plasma phenylalanine levels. Phenylacetate administered in extremely large doses was without effect except to increase the characteristic odor of the urine. A limited toxicity of this compound was noted many years ago in the course of experiments designed to test the limit of synthesis of glutamine with which this compound is conjugated. Massive doses of phenylacetate several times the amount formed by phenylketonuric subjects were repeatedly taken by volunteer subjects with discomfort but with no truly alarming symptoms [108-109]. Most of the other metabolites have been administered at one time or another without untoward effects.

It has been suggested that the lower than normal level of serotonin in the blood of the c patients which is presumably due to impaired synthesis from 5-hydroxytryptophan might contribute to the mental deficiency in phenylketonuric patients [90]. The possibility of administering 5-hydroxytryptophan which can pass the blood brain barrier to make up for a deficiency was suggested.

Pharmacologic studies of *o*-tyrosine the presumed precursor of *o*-hydroxyphenylacetic acid are of considerable interest. *o*-Tyrosine passes the blood brain barrier and is decarboxylated to *o*-tyramine. The latter is concentrated in the brain and the liver and its concentration can be still further elevated by iproniazid which inhibits its oxidation to *o*-hydroxyphenylacetic acid. The effects of this on animals are marked central hyperactivity and sympathetic excitation [97]. The precursors of this metabolite have not been found in phenylketonuric tissues but very small amounts could exert pharmacologic effects and could be missed by the usual analyses.

#### ORIGIN OF METABOLITES

Figure 10-7 indicates by double arrows the major metabolic pathways of phenylalanine, tyrosine and tryptophan under normal conditions. As a consequence of the block in the normal conversion of phenylalanine to tyrosine, phenylalanine must detour through the other little used but not abnormal pathways as indicated. Some metabolites of tyrosine and tryptophan are also diverted along unusual pathways.

It has been suggested that there is inhibition of some of the steps in the normal metabolism of tryptophan and of tyrosine in phenylketonuria. The evidence is indirect but there is indeed an accumulation of derivatives of the  $\alpha$  keto acids of phenylalanine, *o*-tyrosine, tyrosine and tryptophan. The keto acids are the parent substances which can give rise to the standard series of metabolites already listed: the related pyruvic, lactic, acetic and conjugated acetyl form. Knowledge of the individual enzyme reactions on which this metabolic map (Fig. 10-7) is based is

alanine levels with the amount of phenylpyruvic acid excreted [20] and also with the amount of *o*-hydroxyphenylacetic acid excreted [91]. Phenylalanine excretion is also proportional to its plasma level [10, 102]. It is probably true for all of the metabolites that their excretion rate in the urine is proportional to plasma phenylalanine over certain ranges.

The urinary concentrations of three compounds—phenylpyruvic acid, *o*-hydroxyphenylacetic acid, and phenylalanine—have been tested as convenient indicators of the plasma phenylalanine concentration during treatment of patients. Phenylpyruvic acid excretion ceases at phenylalanine plasma levels below 0.6  $\mu$ moles per ml (10 mg per 100 ml) [106] or 0.90  $\mu$ moles per ml (15 mg per 100 ml) [5]. The latter value is probably high; *o*-Hydroxyphenylacetic acid excretion was detected at plasma phenylalanine levels above 0.18 or 0.67  $\mu$ moles per ml (8 or 11 mg per 100 ml) [106, 107]. *o*-Hydroxyphenylacetic acid may therefore be slightly superior as a measure of plasma phenylalanine, but at the critical lower levels excretion of phenylalanine itself may be superior to either acid [106].

#### *Quantitative Relations of Metabolites to Intelligence*

For the vast majority of patients there exists no correlation between intelligence and amounts of any of the metabolites measured. But it must be remembered that chemical measurements have generally been made on phenylketonuric patients of all ages and not at the time when the mental damage was done.

Tabulated plasma phenylalanine levels and I Q's for 18 patients [86] for another 18 patients [87] and for 34 patients [91] show that there is no relationship. There was also no relation between I Q's of 8 patients and their cerebrospinal fluid levels of phenylalanine [86]. Jervis [88] found that the higher plasma phenylpyruvic acid levels were associated with higher I Q's and with better nutritional states in 34 patients. He attributed the low levels of the severely defective patients to their chronic malnutrition. *o*-Hydroxytryptamine levels were independent of the I Q's of 9 patients [90].

Since no relation between intelligence as measured by the I Q test and plasma phenylalanine concentrations exists, none would be expected with the urinary metabolites that are formed in proportion to the plasma phenylalanine. This has been borne out in a number of studies [49, 64, 88, 90, 91].

#### *Pharmacology of Metabolites*

Phenylketonuric patients under treatment with a low phenylalanine diet have been used to a limited extent to test the pharmacologic effects of the various metabolites. Only phenylalanine (or phenylpyruvate) administration produced definite effects on these patients. These were slight ataxia and deterioration of behavior and reappearance or worsening

described below. Each step in the map has been numbered for convenience in discussion.

An alternative pathway of phenylalanine degradation which has not been investigated in phenylketonuria is that giving benzoic acid by a cleavage reaction [110]. This should result in increased hippuric acid excretion. Armstrong, Chao, Iarker and Wall demonstrated this conversion in rats [111].

### *Phenylalanine Hydroxylase (A1)*

The original isotopic demonstration of the phenylalanine hydroxylase reaction was in rats. Deuterium labeled phenylalanine was metabolized to labeled tyrosine isolated from the tissue proteins [112]. The observation was then repeated in phenylketonuric patients with  $C^{14}$  phenylalanine [113]. Less than 10 per cent of the usual amount of tyrosine was formed from phenylalanine. The small amount formed may be attributed to other unknown reactions or to residual activity of the genetically distorted enzyme protein. Residual activity has not been detected in studies of the enzyme in phenylketonuric liver. Direct studies of the enzyme from patients were first reported by Jervis [5] and later by others [114-116].

Phenylalanine hydroxylase assays in liver from normal controls and a total of seven phenylketonuric patients have proved that the enzyme is present in specimens from normal man but that there is no detectable activity in phenylketonuric liver. The enzyme requires for activity a second protein fraction which is widely distributed in the body, either reduced DPN or TPN, and a new coenzyme related to folic acid (see below). All the cofactors are present in phenylketonuric liver. Lack of phenylalanine hydroxylase activity is referable to a fault of the specific enzyme itself. It has not yet been determined whether an altered protein is present in place of the normal enzyme protein or whether the genetic defect is a gene deletion.

Phenylalanine hydroxylase occurs only in the liver. It is found in the liver only after the biochemical differentiation following birth [117]. Its reaction has the following stoichiometry:



Oxygen is required for the reaction. One atom is used in the *p*-hydroxylation and one is simultaneously reduced to water by the associated oxidation of the reduced pyridine nucleotide. The reaction is therefore one of a newly recognized group of oxygenations in which gaseous oxygen is incorporated directly into the substrate. A necessary coenzyme which has not been identified can be replaced by tetrahydrofolic acid although the latter does not function catalytically [118]. The reaction is specific for *L*-phenylalanine. It does not hydroxylate other aromatic compound nor

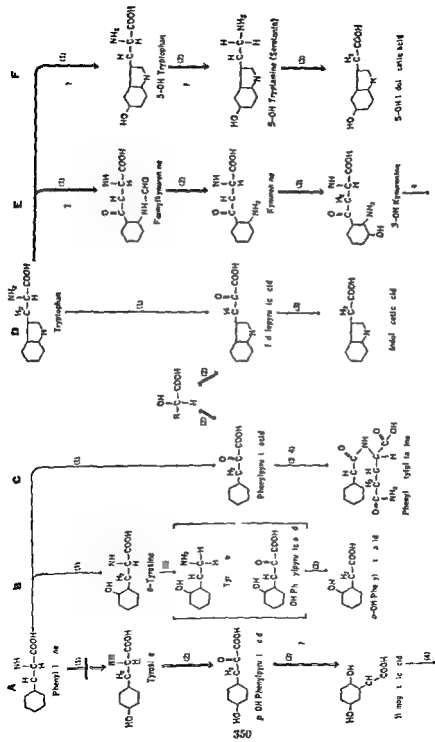


Fig. 10.7 Map of the main normal metallo routes of phenylalanine tyrosine and tryptophan (to the right) the location of the amino acid in the protein (to the left) and the amino acid in the protein (to the left).

details are given in Chap 12 Alcaptonuria The final products are fumaric and acetoacetic acids which can be further oxidized in the citric acid cycle

*Conversion of Phenylalanine to o-Tyrosine o-Tyramine and o-Hydroxy phenylacetic acid (B1 2 and 3)*

o-Hydroxy phenylacetic acid is formed from phenylalanine The former compound appears in the urine labeled with  $C^{14}$  after administration of phenylalanine  $C^{14}$  [129] and the amount excreted is proportional to the concentration of phenylalanine in the plasma [91 107] The two possible pathways of formation are shown in the figure It is most probable that *ortho*-hydroxylation occurs first to form o-tyrosine The enzyme has not been identified o-Tyrosine is excreted as o-hydroxyphenylacetic acid [130] An alternate route through the pyruvate derivative was demonstrated by Hatan [131] Decarboxylation of o-tyrosine to o-tyramine by guinea pig kidney and rat liver extracts was demonstrated by Blaschko [132] This occurs slightly less rapidly than decarboxylation of dihydroxy phenylalanine (dopa) Both reactions may be catalyzed by the dopa decarboxylase If so the suggestion will have to be modified that the latter enzyme is inhibited by metabolites in phenylketonuria and that epinephrine synthesis is decreased in this way [133] The oxidation of o-tyramine by amine oxidase was described by Randall [134] The pathway from o-tyrosine through o-tyramine to o-hydroxyphenylacetic acid was demonstrated in animals by Vitoma et al [97] At present it can only be said that both routes are possible

*Phenylalanine Transaminase (C1)*

The suggestion that phenylpyruvate in phenylketonuria comes from transamination of phenylalanine was first made by Utena and Suto [20] They administered glutamic acid to their patient and noted that the amount of phenylpyruvic acid excreted was decreased This could be explained by reversal of the phenylalanine transaminase reaction



Meister et al [103] have also reported inhibition of phenylpyruvate excretion after administration of glutamic acid or glutamine to two phenylketonuric patients These observations also implicated transamination in the origin of phenylpyruvate

Like the two tyrosine transaminases there are two phenylalanine transaminases which require  $\alpha$  ketoglutarate and pyruvate respectively Both require pyridoxyl phosphate as the coenzyme and both are in highest concentration in liver [123 124 135] The observation that the phenylalanine transaminase appears only after birth in rat liver [136]



does it hydroxylate in the *ortho* or *meta* positions. A brief review of this and related enzymes can be found elsewhere [119].

A nonenzymatic system of ferrous ion, a carbonic acid, a chelating agent and oxygen will hydroxylate phenylalanine [120]. It has been suggested that *o*-tyrosine as well as a little tyrosine might be formed in this way in phenylketonuric patients [121-122] but there is no proof that such a nonenzymatic reaction plays a role in metabolism.

### *Tyrosine Transaminase (12)*

L-Tyrosine is first converted to its keto acid in the course of its oxidation by transamination [123]. There are two specific tyrosine transaminases, one reacting with  $\alpha$ -ketoglutarate and one with pyruvate as acceptors [124]. Both require pyridoxal phosphate. The former enzyme has been partially purified [125]. It does not react with other aromatic amino acids. The liver enzyme, tyrosine  $\alpha$ -ketoglutarate transaminase, is induced to greatly increased levels within a few hours by administration of the substrate or hydrocortisone [126]. Small amounts of the enzyme also occur in the kidney.

### *p-Hydroxyphenylpyruvate Oxidase (13)*

This unusual enzyme catalyzes the first oxidative step in the main metabolism of tyrosine. It occurs in liver and in smaller amounts in kidney. The reaction is also an oxygenation and forms homogentisate without intermediates (2,5-dihydroxyphenylpyruvate is not formed). Ring hydroxylation and migration and oxidative decarboxylation of the side chain apparently occur together.



The enzyme has been partially purified and extensively studied [127-128]. A point of considerable interest is the inactivity of the reaction in *corbutic* animals. The *c* animals excrete *p*-hydroxyphenylpyruvate when fed tyrosine. Ascorbic acid or certain other compounds are required to prevent reaction product inactivation of the enzyme *in vitro*.

The enzyme is completely inhibited by 0.002 M phenylpyruvate. It is the enzyme most sensitive to inhibition by the metabolites of phenylketonuria that has been described [128]. Nevertheless *p*-hydroxyphenylpyruvate is not excreted in large amounts in phenylketonuria even after large doses of tyrosine. The concentration of phenylpyruvate in plasma in phenylketonuria is one fiftieth of the concentration which inhibits *p*-hydroxyphenylpyruvate oxidation.

### *Homogentisate Oxidase (14)*

The enzymes responsible for homogentisate oxidation and the subsequent reactions of tyrosine degradation are all known [123]. Further

as coenzyme. The activity in liver of the more active enzyme which requires  $\alpha$ -ketoglutarate is controlled by hydrocortisone [13].

The enzymatic mechanisms for the further conversion of the indole compounds to indoleacetic acid and its conjugates are unknown.

### *Tryptophan Oxidation (I F)*

Ring oxidation of tryptophan takes place either by opening of the ring to form kynurenine and ultimately nicotinic acid [141] (E4) or by introduction of the 5 hydroxy group in the serotonin series [142]. Only the 5 hydroxylation of tryptophan itself remains to be described in enzymatic terms (I I). The largest amount of tryptophan is oxidized by opening of the indole ring to form formylkynurenine [143] (F1). This enzyme now called tryptophan pyrrolase is another which catalyzes an oxygenation reaction. It is not known to be inhibited by any of the metabolites of phenylketonuria.

5-Hydroxytryptophan decarboxylase (F2) is a specific pyridoxal requiring enzyme found in a number of tissues including liver and brain. It has been suggested that the low level of serotonin in the blood of phenylketonuric patients arises from depressed activity of this enzyme [144]. Davison and Sandler [145] found that the enzyme was inhibited 50 per cent by 0.02M phenylpyruvic acid and somewhat less by other metabolites of phenylketonuria. This concentration is 500 times the amount of phenylpyruvic acid found in the blood in phenylketonuria (0.013  $\mu$ moles per ml).

### *Summary of the Origin of Metabolites*

The inactivity of the phenylalanine hydroxylase is firmly established as the primary defect in phenylketonuria. The minor metabolic pathways employed by phenylalanine in detouring around this metabolic block are well outlined. The various enzymatic reactions are known with the exception of the reaction leading to the *o*-hydroxyphenyl series and the reactions for interconverting a few of the minor metabolites. Uncertainty exists only about the origin of defects in pathways unrelated to phenylalanine. Inhibition by phenylpyruvic acid or its derivatives of the degradation of tryptophan and tyrosine and the formation of serotonin may occur but the evidence is indirect. In addition to the enzyme inhibitions cited above Bickel, Kennedy and Quastel [146] have demonstrated inhibition by phenylalanine both of tyrosine degradation and of tyrosine incorporation into proteins by tissue slices.

Inhibitions of these sort may be possible explanation of some of the abnormalities which are therapeutically reversible in phenylketonuria but so also may be adaptive changes in the metabolic process brought on by the unusual chemical milieu of the tissues. For example, a general increase in the rate of transamination of tyrosine and tryptophan with

may be relevant to the finding that excretion of phenylpyruvate may be delayed until 1 month after birth in phenylketonuria [134]

### *Lactic Dehydrogenase (C2)*

Lactic dehydrogenase catalyzes the reversible reduction of  $\alpha$  keto acids by  $\text{DPNH}$  to their lactic acid derivatives. It is undoubtedly responsible for the appearance of the lactic derivatives of the  $\alpha$  keto metabolites in phenylketonuria. The specificity of this enzyme is low. Meiter [135] demonstrated that it reacts with phenylpyruvate, *p*-hydroxyphenylpyruvate and indolepyruvate at about one-one hundredth the rate of its reaction with pyruvic acid. In view of the large amounts of this enzyme in the body, the relatively slow rates are adequate to account for the quantities of lactic acid derivatives which are formed.

### *Phenylacetate Formation (C3)*

The mechanisms for the conversion of aromatic  $\alpha$ -keto acids to the acetic acid derivatives are not known. The reactions are analogous in type to the oxidative decarboxylations of pyruvate to acetate or of  $\alpha$ -ketoglutarate to succinate.

### *Phenylacetylglutamine Synthesis (C4)*

This conjugation product is formed only by man and the higher apes. The enzymatic synthesis is similar to that involved in protein formation. Moldave and Meister [136] showed that the synthesis occurs by activation of phenylacetate with  $\text{ATP}$ , then transfer of the phenylacetyl group, first to  $\text{CoA}$ , and then to glutamine.



Phenylacetylglutamine is the only one of the characteristic metabolites of phenylketonuria whose formation has been measured in the tissues of a phenylketonuric patient. Meister [140] found that a liver biopsy sample from a phenylketonuric patient formed phenylacetylglutamine approximately five times faster than the rate observed in liver preparations from patients without phenylketonuria. This suggests that the enzyme is adaptive in nature and indicates one way in which the body may adjust to handle unusual amounts of metabolites by alternate metabolic routes.

### *Tryptophan Transaminases (D1)*

Like the other transaminations mentioned, this reaction is also catalyzed by two specific enzymes, one using  $\alpha$  ketoglutarate and the other pyruvate as the acceptors. The enzymes require pyridoxal phosphate

acid level in plasma (Table 10-7) is one hundredth this concentration (0.013  $\mu$ moles per ml or 0.000013 M) the possibility of some *in vivo* inhibition of this enzyme is remote. If this enzyme is also involved in the formation of *o*-hydroxyphenylacetic acid by decarboxylation of *o*-tyrosine it might also be competitively inhibited.

### *$\beta$ -Globulins of Serum and Hemoglobin Synthesis*

Electrophoretic studies of serum in 17 cases of phenylketonuria have revealed multiple abnormal sharp high peaks in the  $\beta$  globulin fraction in 8 cases [87]. This fraction contains the  $\beta$  lipoproteins. Similar findings were encountered in one case of active hepatitis. The abnormal components disappeared in those patients treated with a low phenylalanine diet and reappeared when phenylalanine was restored to the diet. Thus the high phenylalanine concentration is the factor responsible for the abnormal  $\beta$  globulin components.

The possibility of an error in the synthesis of one protein, hemoglobin, in the presence of the high concentration of phenylalanine was excluded by the precise analysis by Allen and Schroeder [103]. The incorporation of phenylalanine into hemoglobin by a phenylketonuric subject was correct to within less than one residue per molecule. Thus protein synthesis may be remarkably accurate even in abnormal conditions, but it is possible that other proteins in phenylketonuria might be formed with an excess phenylalanine content.

### *Cerebral Oxygen Consumption*

Oxygen arteriovenous differences in the cerebral circulation were lower in nine phenylketonuric patients than in other comparable mentally defective patients [164, 165]. Since blood flow measurements were not made it is not known whether cerebral oxygen uptake was actually decreased. The oxygen uptake per gram may still be normal because of the reduced brain weight of these patients.

### *Altered Pattern of Plasma Amino Acids*

The abnormalities of the biochemical milieu in which the cells of phenylketonuric patients must live and grow have not been defined as well as might be. Knowledge of the nutritional requirements at the cellular level is inadequate to predict the consequences of these abnormalities. One example of the potentially harmful alterations of the cellular environment is the distorted pattern of free amino acids. The  $\alpha$ -amino nitrogen of the phenylalanine found in this disease is about half of the normal total  $\alpha$ -amino nitrogen of plasma, yet the total  $\alpha$ -amino nitrogen in phenylketonuric plasma is not substantially higher than normal [2]. Obviously the concentrations of other amino acids must be depressed.

formation of excess proportions of their  $\alpha$  keto acids would equally well account for the observed abnormalities in metabolism of these compounds. The question can be settled only by measurements of the reactions in question under physiologic conditions in the phenylketonuric patient.

### OTHER BIOCHEMICAL ABNORMALITIES

#### *The Conversion of Tyrosine to Melanin Pigment*

The decreased pigmentation in phenylketonuria is accounted for by competitive inhibition of phenylalanine on the tyrosinase system. Danes and Balis [147] first demonstrated competition between phenylalanine and tyrosine with a mushroom tyrosinase system. Miyamoto and Fitzpatrick [148] confirmed this with mammalian tyrosinase. An unusually clear demonstration of the phenomenon of competitive inhibition under physiologic conditions is provided by the darkening of new grown hair of phenylketonuric patients when either tyrosine intake is increased [149] or phenylalanine intake is lowered [5].

#### *Epinephrine Tolerance*

There are suggestions that phenylketonuric patients are deficient in epinephrine. A test involving serial blood pressure measurements following injections of Mecholyl and of epinephrine has been used to determine the autonomic reactivity of psychiatric patients [150]. Cawte [151] found that two phenylketonuric patients reacted like the controls to Mecholyl but had an increased hypertensive response to epinephrine. This finding was later supported [152] by studies on 7 phenylketonuric patients. Only 7 of 30 phenylketonuric subjects were able to give regularly the necessary cooperation. Those selected had on the average half again as high a rise in blood pressure as a group of 9 nonphenylketonuric mentally defective control patients and they required a longer period for return to basal values after epinephrine injection. The difference between the two groups is statistically significant. Cawte related this responsiveness of phenylketonuric patients to their tense reactive attitude. Another explanation is found in Cannon's rule that a neuroreceptor deprived of its usual stimulus becomes more sensitive. Plasma concentrations of epinephrine were actually found to be lower than normal in 13 phenylketonuric children but they were also low in patients with other kinds of mental defect [96]. Measurement of the epinephrine in adrenal glands from one patient showed a normal or high value [121].

The inhibition of dopa decarboxylase by metabolites of phenylketonuria has been suggested as an explanation of the low epinephrine plasma value (or increased sensitivity to epinephrine) [77]. Phenylpyruvate and lactate are the most effective inhibitors. They have a pronounced effect in concentrations as low as 0.003*M*. Since the average phenylpyruvic

acid level in plasma (Table 10 7) is one hundredth this concentration ( $0.043 \mu\text{moles per ml}$  or  $0.000043 M$ ) the possibility of some *in vivo* inhibition of this enzyme is remote. If this enzyme is also involved in the formation of *o*-hydroxyphenylacetic acid by decarboxylation of *o* tyrosine it might also be competitively inhibited.

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Only one analysis of plasma amino acids in phenylketonuria has apparently been done. This is referred to briefly by Stein and Moore [57]. The values of the amino acids in this study (per oral communication from W. H. Stein and S. Moore) appear in Fig. 10-8 and are compared with the average plasma values from five fasting adult males obtained by

#### AMINO ACIDS

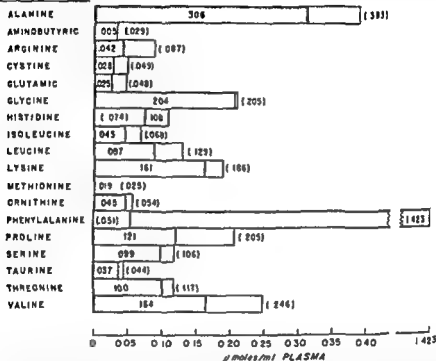


Fig. 10-8 Amounts of some of the free plasma amino acids in a 33 year-old male phenylketonuric patient (I.Q. 44) (shaded column) superimposed on the mean values for five normal adult males (re calculated and drawn from data of Stein and Moore [57] and personal communications). It appears that the high level of phenylalanine may depress the levels of most other amino acids with maintenance of a normal value for the total  $\alpha$  amino nitrogen.

column chromatography by the same authors. The high phenylalanine evidently caused a depression of the concentration of most other amino acids. Glycine and histidine are unique in being present at a normal and an increased level respectively. Of the undetermined amino acids only glutamine normally occurs in significant amounts and it also may be changed in phenylketonuria.

#### Renal Clearance

The renal clearance of phenylalanine in both the dog [104] and man [105] has been determined. Like the majority of amino acids phenyl

alanine is well reabsorbed. Clearance was about 1 to 3 ml per min. At normal blood concentrations only about 1 per cent of the filtered phenylalanine appears in the urine but as much as 15 per cent of that filtered appears in the urine when the concentration in the serum is raised tenfold. A  $T_m$  (tubular secretion max.) cannot be reached with the plasma phenylalanine levels obtainable in normal persons but it would appear from the studies above that the  $T_m$  would be exceeded at slightly higher levels than those studied. No measurements of renal clearance have been reported in phenylketonuric patients. Here the blood levels obtainable would permit the direct measurement of the  $T_m$ .

### *Experimental Phenylketonuria*

An experimental approach to this disease was opened by Auerbach et al [156, 157] who found that phenylalanine hydroxylase synthesis is repressed by administration of tyrosine, the product of the reaction. This phenomenon which is the reverse of substrate induction of an enzyme had not previously been known in animals although it had been observed in microorganisms. Phenylalanine hydroxylase activity was reduced to one twentieth of its usual value by maintaining young rats on a 5 per cent tyrosine diet. The addition of extra phenylalanine to this diet did not alter the enzyme activity since the enzyme is not adaptively increased by its substrate. The excretion of phenylpyruvic acid by the animals was high but this may have been because DL-phenylalanine was given. However the plasma level of L-phenylalanine was lightly raised and other tests suggested that the treated animals showed some retardation of learning ability. These experiments are an ingenious duplication by environmental influences of the metabolic picture produced by an hereditary enzyme defect.

## GENETICS

Familial cases appeared among the first reported patients with phenylketonuria and perhaps this was responsible for the initial recognition of the disease. There were three sib pairs among Gilling's first 10 patients. It is reported [158] that the mother of two of the original patients struck by their similarities and particularly by their malodorous urines insisted that special biochemical observations be made.

The genetics of phenylketonuria is now precisely known. Jervis [12] had this to say of the factors facilitating the genetic study: 1) the identification of this condition is simple and exact being made by a chemical test; 2) the character segregates sharply affected individuals being entirely different biochemically from nonaffected ones; 3) the disease fulfills the requirement of being a unit in a biological (biochemical) sense. It should be added that the disease is rare for this permits some



Only the analysis of the acid-fastness of the bacteria is of interest. The results are shown in Table 1. The results of the analysis of the acid-fastness of the bacteria are shown in Table 1. The results of the analysis of the acid-fastness of the bacteria are shown in Table 1.

# AMINO ACIDS

| AMINO ACID    | 300 | 350 |
|---------------|-----|-----|
| ALANINE       | 100 | 100 |
| ASPARAGINE    | 100 | 100 |
| ASPARTIC ACID | 100 | 100 |
| GLUTAMINE     | 100 | 100 |
| GLUTARIC ACID | 100 | 100 |
| GLUTAMIC ACID | 100 | 100 |
| GLYCINE       | 100 | 100 |
| ISOLEUCINE    | 100 | 100 |
| LEUCINE       | 100 | 100 |
| LYSINE        | 100 | 100 |
| METHIONINE    | 100 | 100 |
| ORNITHINE     | 100 | 100 |
| PHENYLALANINE | 100 | 100 |
| PROLINE       | 100 | 100 |
| SERINE        | 100 | 100 |
| THreonine     | 100 | 100 |
| VALINE        | 100 | 100 |

TABLE 1. AMINO ACID ANALYSIS OF THE ACID-FAST BACTERIA. The results of the analysis of the acid-fastness of the bacteria are shown in Table 1.

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simplified assumptions in handling the distribution data. These rare cases are largely concentrated into institutions where they can be found observed and reported in sizable groups. Consequently more cases of phenylketonuria are known than of any other comparably rare disease.

### MODE OF INHERITANCE

The first large scale genetic analysis by Jervis [3] comprised 213 patients and indicated that the disease is transmitted by a single autosomal

TABLE 10-9 FREQUENCY OF PHENYLKETONURIC OFFSPRING IN VARIOUS MATINGS

| Marital partners                  |                          |                                   |                          | Theoretic frequency of affected children if both partners were carriers | Chances of affected children from such a mating |
|-----------------------------------|--------------------------|-----------------------------------|--------------------------|---|---|
| A                                 |                          | B                                 |                          |   |   |
| Carrier status                    | Chances of carrying gene | Carrier status                    | Chances of carrying gene |   |   |
| Unknown                           | 1/80                     | Unknown                           | 1/80                     | 1/4   | 1 in 600  |
| Unknown                           | 1/80                     | Normal sibling of phenylketonuric | 2/3                      | 1/4   | 1/480   |
| Unknown                           | 1/80                     | Parent of phenylketonuric         | 1                        | 1/4   | 1/320   |
| Unknown                           | 1/80                     | Phenylketonuric                   | 1                        | 1/2   | 1/160   |
| Normal sibling of phenylketonuric | 2/3                      | Normal sibling of phenylketonuric | 2/3                      | 1/4   | 1/9   |
| Normal sibling of phenylketonuric | 2/3                      | Parent of phenylketonuric         | 1                        | 1/4   | 1/6   |
| Normal sibling of phenylketonuric | 2/3                      | Phenylketonuric                   | 1                        | 1/2   | 1/3   |
| Parent of phenylketonuric         | 1                        | Parent of phenylketonuric         | 1                        | 1/4   | 1/4   |
| Parent of phenylketonuric         | 1                        | Phenylketonuric                   | 1                        | 1/2   | 1/2   |
| Phenylketonuric                   | 1                        | Phenylketonuric                   | 1                        | 1   | 1   |

Calculated on prevalence rate of disease as approximately 1/100,000 as suggested by Jervis

SOURCE: S. W. Wright et al. [10]

recessive gene. His definitive review [12] included data from 146 personally observed families: 46 families of Munro [17], 22 families of Polling-Mohr and Ruud [10], and 52 other families reported in the world literature up to 1934. In the 266 families were 1,094 siblings: 433 (39.6 per cent) with phenylketonuria. On the hypothesis of a single autosomal recessive gene, 25 per cent would be affected. Corrections for the method of ascertainment by the methods of Weinberg and Lenz, which allowed for the uncounted families with only normal children, gave corrected

percentages with standard errors of affected children as  $27.37 \pm 2.17$  (sib method)  $22.48 \pm 2.66$  (proband method) and within one third of the standard error of the hypothetical 25 per cent by the Lenz *a priori* method. There was no significant difference in the sex incidence. The evidence for autosomal recessive transmission was further substantiated by the occurrence of parental consanguinity in 8.33 per cent of 206 of these

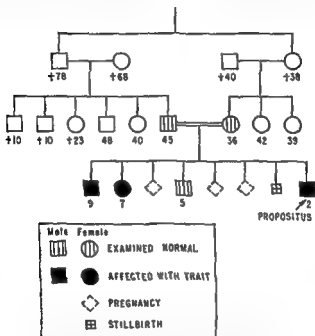


Fig 10-1 Pedigree of Japanese family with phenylketonuria illustrating parental consanguinity, parental normality, and multiple siblings affected [27]. Age in years and deaths (+) are indicated. Other symbols are standard.

families from the United States, England, and Norway. This is the incidence expected for a recessive disease occurring once in 25,000 times in a population that has approximately 1 per cent cousin marriages. The precision with which the inheritance of phenylketonuria conforms to expectations lends considerable weight to the simple mathematic calculation of the risk of this disease in different types of matings, as shown in Table 10-9.

In the vast majority of families the parents are normal, are often related, and rarely have affected relatives. A typical pedigree is shown in Fig 10-9. It is probable that in the few twin pairs reported the monozygotic pairs are concordant while the dizygotic pairs are either concordant or discordant, as expected (Table 10-10). Sixty-five half sibs of affected

simplified assumptions in handling the distribution data. These rare cases are largely concentrated into institutions where they can be found observed and reported in sizable groups. Consequently more cases of phenylketonuria are known than of any other comparably rare disease.

### MODE OF INHERITANCE

The first large scale genetic analysis by Jervis [3] comprised 213 patients and indicated that the disease is transmitted by a single autosomal

TABLE 10-9 FREQUENCY OF PHENYLKETONURIC OFFSPRING IN VARIOUS MATINGS

| Marital partners                  |                          |                                   |                          | Theoretic frequency of affected children if both partners were carriers | Chances of affected children from such a mating |
|-----------------------------------|--------------------------|-----------------------------------|--------------------------|---|---|
| A                                 |                          | B                                 |                          |   |   |
| Carrier status                    | Chances of carrying gene | Carrier status                    | Chances of carrying gene |   |   |
| Unknown                           | 1/80                     | Unknown                           | 1/80                     | 1/4   | 1/2500  |
| Unknown                           | 1/80                     | Normal sibling of phenylketonuric | 2/3                      | 1/4   | 1/480   |
| Unknown                           | 1/80                     | Parent of phenylketonuric         | 1                        | 1/4   | 1/320   |
| Unknown                           | 1/80                     | Phenylketonuric                   | 1                        | 1/2   | 1/160   |
| Normal sibling of phenylketonuric | 2/3                      | Normal sibling of phenylketonuric | 2/3                      | 1/4   | 1/9   |
| Normal sibling of phenylketonuric | 2/3                      | Parent of phenylketonuric         | 1                        | 1/4   | 1/6   |
| Normal sibling of phenylketonuric | 2/3                      | Phenylketonuric                   | 1                        | 1/2   | 1/3   |
| Parent of phenylketonuric         | 1                        | Parent of phenylketonuric         | 1                        | 1/4   | 1/4   |
| Parent of phenylketonuric         | 1                        | Phenylketonuric                   | 1                        | 1/2   | 1/2   |
| Phenylketonuric                   | 1                        | Phenylketonuric                   | 1                        | 1   | 1   |

Calculated on prevalence rate of disease as approximately 1/100,000 as suggested by Jervis

SOURCE: S. W. Wright et al. [10]

recessive gene. His definitive review [12] included data from 146 personally observed families: 46 families of Munro [17], 22 families of Folling-Mohr, and Ruud [10] and 52 other families reported in the world literature up to 1954. In the 266 families were 1,094 siblings: 433 (39.6 per cent) with phenylketonuria. On the hypothesis of a single autosomal recessive gene, 25 per cent would be affected. Corrections for the method of ascertainment by the methods of Weinberg and Lenz, which allowed for the uncounted families with only normal children, gave corrected

of members of affected families have been recorded in a search for linkage of the blood groups with the gene for phenylketonuria [17 38 160]. If such linkage exists at all it is loose and not close enough to identify the heterozygotes. Psychoses were suggested as more frequent among parents and other relatives of phenylketonuric patients but statistical studies have not confirmed (or denied) this [3 17]. The possibility deserves further study with the aid of the chemical tests recently developed for identifying heterozygotes.

### *Chemical Identification of Heterozygotes of Phenylketonuria*

With Jervis' direct proof that phenylalanine hydroxylase activity is missing from the livers of patients with phenylketonuria [8] and his final proof that this is a recessive disease [12] it was considered possible that the heterozygous carriers with one defective gene would have less than the normal complement of phenylalanine hydroxylase as suggested by C. G. King [161] during the discussion following the presentation of Jervis' classical review. Tölling and Closs [162] hinted that there might be increased phenylalanine in the urine of heterozygotes. Waelsch could find no abnormality in phenylalanine level in the blood in 10 pairs of parents [163].

This question was reinvestigated by Hsia, Driscoll, Troll, and Knox [9] who gave standard doses of L-phenylalanine and followed the plasma concentration of phenylalanine by a specific enzymatic method. Parents of known phenylketonuric patients were the heterozygotes. Their phenylalanine concentrations were higher and more sustained after the given dose than those of control individuals. The results are shown graphically in Fig. 10-10. The data from this study were published in detail by Hsia and Paine [164]. The same distinction between 10 heterozygotes and 10 controls was confirmed by Berry, Sutherland, and Guest [80]. They measured serum phenylalanine by a chromatographic method which gave about 30 per cent lower values than the specific enzymatic method but it revealed the same relative difference between known heterozygotes and controls (Fig. 10-11).

Further investigation disclosed that more detailed measurements could detect a subtle abnormality even under basal conditions. Knox and Mesinger [60] refined the specific enzymatic method to determine more precisely the normal levels of phenylalanine and were able to show that a group of heterozygotes had a significantly higher mean fasting level than a control group (Fig. 10-12). There was the same degree of separation between the group by this test as by the tolerance test. The phenylalanine concentration of the heterozygote group was about 1.5 to 2.5 times the normal both in the fasting state and after the loading dose.

The tolerance test therefore did not significantly enhance a latent abnormality nor did it reveal a deficient metabolic reserve since a

individuals were all normal as expected except for two whose father had married two sisters and produced a phenylketonuric offspring in each union [12]

Records of matings of affected individuals to normal individuals exist (Table 10-11). The phenylketonuric offspring in some of these matings

TABLE 10-10 PHENYLKETONURIA IN TWINS

| Reference                                  | Zygosity                     | Phenylketonuria | Normal       |
|--|------------------------------|-----------------|--------------|
| [3] Jervis 1939 Cases 185-186              | Monozygous                   | 2               |              |
| [3] Jervis 1939 Cases 21-22                | Monozygous (?)               | 2               |              |
| [18] Wright and Tarjan 1957                | Monozygous                   | 2               |              |
| [3] Jervis 1939 Cases 158-159              | Dizygous                     | 2               |              |
| [3] Jervis 1939 Case 1                     | Dizygous                     | 1               | 1            |
| [189] Thompson 1957                        | Not determined<br>both males | 1               | 1            |
| [51] Woolf et al 1958                      | Dizygous                     | 1               | 1            |
| [10] Felling Mohr and Ruud<br>1944 Case 11 | Dizygous (?)<br>females      | 1               | 1 (probably) |

TABLE 10-11 MATINGS OF PHENYLKETONURIC INDIVIDUALS

| Reference | Phenylketonuric parent       | Other parent            | Offspring                     |
|-----------|------------------------------|-------------------------|-------------------------------|
| [12]      | Female (?)                   | (Illegitimate union)    | 1 normal                      |
| [81]      | Female low grade             | (Illegitimate union)    | 1 probably normal<br>idiot    |
| [10]      | High grade married<br>female | Normal                  | 2 normal                      |
| [9]       | Female imbecile Case<br>24   | Normal (heterozygote ?) | 2 normal<br>2 phenylketonuric |
| [98]      | High grade married<br>male   | Normal (heterozygote ?) | 1 normal<br>1 phenylketonuric |

can be adequately explained by the heterozygosity of the apparently normal spouses. Births of normal children from phenylketonuric mothers establish the fact that no intrauterine damage is done to the fetus by the metabolic abnormalities of the phenylketonuric mother. The corollary that a phenylketonuric fetus would not harm himself by his own metabolites during intrauterine life is the basis for the view that phenotypic defects of the phenylketonuric child develop after birth.

#### HETEROZYGOTE CHARACTERISTICS

The concept of the recessivity of phenylketonuria is derived from failure to recognize any constant abnormality of the heterozygous carriers of the gene. Chemical and intelligence test results of parents have been uniformly negative. Data on ABO agglutinogens in the blood

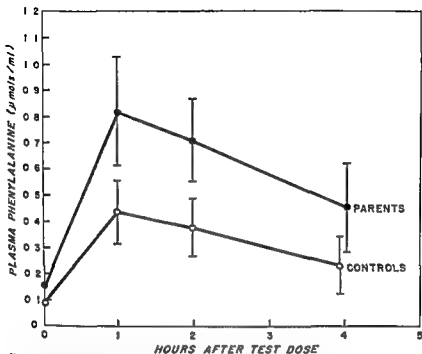


Fig 10-11 Tolerance curves following phenylalanine administration to 10 controls and 10 parents of phenylketonuric patients expressed in the same way as the results in Fig 10-10 (Drawn from results of H. Berry et al [8])

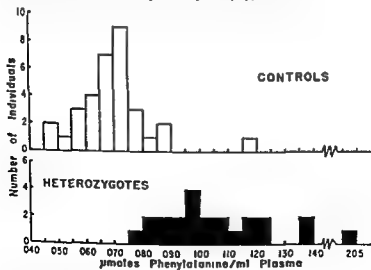


Fig 10-1 Distributions of plasma phenylalanine levels in 33 control and 23 heterozygous individuals [10]



similar degree of abnormality was present even under basal conditions. Only a reduced capacity of heterozygotes to metabolize phenylalanine has been demonstrated but the phenylalanine hydroxylase reaction is the one deficient in heterozygotes because less tyrosine is formed. Jervis [160] has used the rise in tyrosine in plasma after a dose of phenylalanine as a test of heterozygosity. This test is less practical since doses of the order of 25 gm are needed to show that the controls clearly form more tyrosine than do heterozygotes.

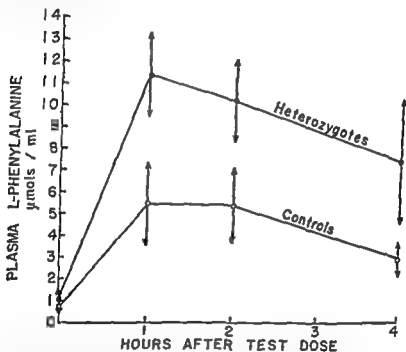


Fig 10-10 Plasma L-phenylalanine levels in normal (—○—) and heterozygous (—●—) persons following oral doses of 0.1 gm L-phenylalanine per kilogram body weight. Each line represents the mean value from 19 persons. The length of the arrows represents one standard deviation above and below the mean [58].

It has been suggested that the altered phenylalanine hydroxylase in phenylketonuria introduces an *ortho*- instead of a *para* hydroxyl group into phenylalanine and in this way gives rise to a qualitatively new (abnormal) *o* hydroxyphenyl series of metabolites [130]. Berry et al [80] found in the course of their tolerance test study that the heterozygote type of blood curve was almost invariably associated with urinary excretion of *o*-hydroxyphenylacetic acid and that no other consistent urinary product was found either in normal or heterozygous individuals. Cullen and Knox [107] found that this substance is excreted by everyone when the plasma level of phenylalanine is sufficiently high. Thus excretion therefore offers no improvement over the usual tolerance test.

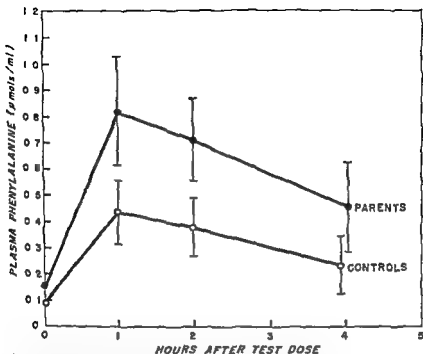


Fig 10-11 Tolerance curves following phenylalanine administration to 10 controls & 10 parents of phenylketonuric patients expressed in the same way as the result in Fig 10-10 (Drawn from results of H. Berry et al [9].)

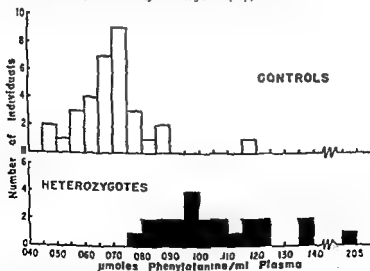


Fig 10-12 Distributions of plasma phenylalanine levels in 33 control and 23 heterozygote individuals [10]

*Genetic Analysis Using the Chemical Test for Heterozygosity*

It would be of great interest and practicality if the genotype of a single individual could be determined from his metabolic phenotype. The overlap between normal persons and heterozygotes in both tests described above was about 15 per cent. A total of 12 apparently normal siblings of phenylketonuric patients was tested in the two studies. Six were judged to be normal and six to be heterozygotes (expected 8.4). Also in each study one control fell well into the heterozygote range. Such a frequency of true heterozygotes in the normal population (2 in 29) would be much higher than the calculated incidence of about 1 in 83. Obvious reasons such as liver disease which might also elevate phenylalanine concentrations, were excluded in these studies. It is probable that other unidentified factors may alter phenylalanine levels. The assignment of a person to one or the other genotype is uncertain with only this kind of information.

*Incidence of Mental Disease in Heterozygotes*

This question has not been resolved in part because of the paucity of known heterozygotes available for study and because the incidence of unidentified phenylketonuric heterozygotes in the population (at least 1 per cent) is roughly comparable to the incidence of mental disease. Heterozygotes could make up a large fraction of mental patients, but a very high susceptibility has doubtless not been overlooked.

Tollings' original report [1] stated that an aunt of one patient and a great aunt of another had dementia praecox. He subsequently noted the incidence of psychoses in later life among relatives of the phenylketonuric patients but doubted that it was higher than was expected in the population as a whole [10]. Penrose was primarily responsible for the further interest in this question. He [160] reported the pedigree of a large family containing four aunts, at least one of whom was phenylketonuric. There were 61 other individuals in the family and of these 6 showed definite signs of insanity, of which the mean age of onset was about 50 years. All of the 6 were carriers of the abnormal gene or at least there was a high probability that they were.

The first controlled study of the incidence of mental disease in phenylketonuric families was that of Munro [17]. Comparison with the incidence in 653 control families of imbecile and idiot patients showed that the incidence of psychoses in the phenylketonuric families was more than twice expectation but barely significant statistically. The incidence of psychoses in the 47 phenylketonuric families increased with the age of the members: the older the relatives, the greater the chance of mental disease. The most striking comparison was between family members

over age 45 where there was twice the expected incidence of mental disease in the phenylketonuric families

Thompson [159] described the relatives of 8 phenylketonuric patients and of 8 low grade mental defectives without phenylketonuria. Mental deficiency or mental disorder of a serious nature was not found in the close relatives of the control patients but mental deficiency or mental disorder was found in each of the phenylketonuric families. Omitting mental deficiency 13 relatives in 6 of the 8 phenylketonuric families had some kind of mild or severe mental disorder. Three relatives in 2 of 8 control families had mild disorder. Just over 100 relatives in each group were considered. Serious disorders in the phenylketonuric families appeared in male relatives much more frequently than in female (1 female and 7 males) and except for 1 who first became psychotic at age 60 years all the disturbed persons had their mental disability throughout life.

If the observed instances of mental disease are referred only to that fraction of the relatives who might be expected to be heterozygotes (e.g. all parents, one half of the grandparents, etc.) the corrected incidence of mental disease in heterozygotes in the families so far studied is of the order of 20 per cent. This of course is a maximal incidence and one that is valid only if heterozygotes do have an increased incidence of mental disease. Because mental disease and heterozygosity for phenylketonuria have similar incidences heterozygosity could account for as much as one fifth of mental disease patients.

An unpublished survey of plasma phenylalanine level in approximately 100 patients in mental hospitals and an equal number of controls reveals a slightly higher mean level in the institutionalized population [160]. The data (Table 10-12) indicate clearly that most mental hospital

TABLE 10-12 MEAN PLASMA PHENYLALANINE LEVEL IN CONTROL AND MENTAL HOSPITAL POPULATION

| Subjects                | No. of subjects | Mean plasma phenylalanine<br>μmoles/l ± s.d. | P (vs control) |
|-------------------------|-----------------|--|----------------|
| Blood donors (controls) | 106             | 0.09 ± 0.015                                 |                |
| Male                    | 80              | 0.08 ± 0.015                                 |                |
| Female                  | 6               | 0.090 ± 0.015                                |                |
| Heterozygotes           | 25              | 0.104 ± 0.016                                | <0.001         |
| Male                    | 19              | 0.109 ± 0.015                                |                |
| Female                  | 13              | 0.100 ± 0.016                                |                |
| Mental patients         | 103             | 0.093 ± 0.018                                | <0.05          |
| Alcoholics              | 24              | 0.098 ± 0.01                                 | <0.01          |
| Nonalcoholics           | 9               | 0.09 ± 0.01                                  | <0.1           |
| Male                    | 43              | 0.096 ± 0.016                                | <0.001         |
| Female                  | 11              | 0.091 ± 0.011                                | 0.4 to 0.5     |

patients are not phenylketonuric heterozygotes. An excess of heterozygotes or some other factor that also gives elevated serum phenylalanine concentrations could account for the higher mean values in the mental hospital population. Elevated phenylalanine values were found 15 per cent more commonly in the mental hospital populations than in the control population. This is near the maximum estimate from the retrospective studies. Some of these higher concentrations occurred in alcoholics in whom liver dysfunction might provide an independent explanation for their high levels. The elevated values were not more common in patients over 40 years nor in a specific diagnostic group but they were significantly more common in male patients than in females. Such a sex difference did not appear in the controls. This recalls Thompson's report of the higher incidence of mental disorder in male relatives of phenylketonuric patients. It can only be concluded that hospitalizable mental disorders might occur in as many as one fifth of heterozygotes of phenylketonuria and more frequently in males than females. A more precise method must be used to prove that mental disorder is more frequent in heterozygotes of phenylketonuria.

## TREATMENT

The therapy of phenylketonuria is considered here only for the confirmation it provides that phenylalanine accumulation is the primary cause of disability in this disease. The earlier and useless treatments employing various types of diets and vitamin, mineral and hormone supplements will not be considered. Attention need be given only to the low phenylalanine regimen. The principle of this treatment is to provide only the amount of phenylalanine needed for growth and repair.

Phenylalanine limitation was first attempted by Dent (unpublished) and by Armstrong and Tyler [8] with diets in which protein was replaced by a costly mixture of pure amino acids. L. I. Woolf (unpublished) suggested a practical method of preparation of casein hydrolyzate with phenylalanine removed by charcoal treatment. Bickel et al. [7] in 1951 maintained a 2 year-old girl for 6 weeks on a diet based on this preparation. Reversal of the major biochemical abnormalities was demonstrated at that time but a larger number of cases and longer periods of treatment have been necessary to evaluate the effect of this diet on the clinical symptomatology. These experiments have been possible only because essentially normal growth and development of infants and children can be maintained on this regimen while at the same time the level of phenylalanine in the body is held at the necessarily normal concentration. Limitation of phenylalanine intake with more normal diets low in protein have not been successful in either lowering the phenylalanine concentrations or maintaining adequate nutrition [62-106].

The most common difficulty encountered in limiting phenylalanine intake is dietary in efficiency with loss of body protein cessation of growth and flooding of the tissues with phenylalanine released from the body protein. In a few cases severe epileptiform seizures were precipitated shortly after the diet was instituted. Adequate control depends upon meeting but not exceeding the phenylalanine requirement and meeting the caloric and other nutritional requirements [167]. Clinical experience suggests that the phenylalanine requirement estimated at 15 mg per kg per day [167] is too low for young children. It may often be two or three times this figure [168-169]. Lang Knopp and Weber [170] recommend the higher intake until age 3. The requirement is met by supplying carefully measured amounts of protein such as milk. Adequate calories must be supplied from essentially nonprotein sources of carbohydrate and fat along with accessory nutrients. The high caloric requirement (more than 100 Cal per kg per day) is apparently associated with the less efficient utilization of free amino acids than of whole proteins. Patients appear to do better if a large part of the calories comes from carbohydrates [55].

The degree of biochemical control must be known to evaluate the results of therapy. Many patients alternate between the extremes of inadequate restriction and too stringent phenylalanine restriction with weight loss or growth failure. Increased phenylalanine plasma concentration and phenylpyruvic acid excretion occur in both situations. Since phenylpyruvic acid excretion occurs only at moderately elevated plasma phenylalanine levels, continued growth without phenylpyruvic acid excretion can be taken to mark the upper limit of acceptable control. Phenylalanine serum elevations short of phenylpyruvic acid excretion may adversely affect the patient. The best regulation is possible only with repeated plasma phenylalanine measurements or possibly measurements of urinary *o*-hydroxyphenylacetic acid or phenylalanine as discussed above under Biochemical Diagnostic Criteria and Methods.

### *Reversal of Biochemical Abnormalities*

All the major biochemical abnormalities of phenylketonuria are reversed by the low phenylalanine diet. Figure 10-13 shows the dramatic effects observed in the first treated case. Also illustrated are preliminary periods of treatment showing the effects of several other dietary regimens. Besides the decrease of phenylalanine and phenylpyruvic acid,  $\alpha$ -hydroxyphenylacetic acid [29], phenylacetylglutamine, phenyllactic acid, indolyl lactic acid, and indolylacetic acid [21] decreased to normal values. The peculiar odor of the urine vanished. There are no reports of the effect of treatment on the supposed excretion of *p*-hydroxyphenyl derivatives or on the sensitivity of these patients to epinephrine. The amount of 5-hydroxyindoleacetic acid in urine was unchanged, but the serotonin in blood rose during treatment [1,5].

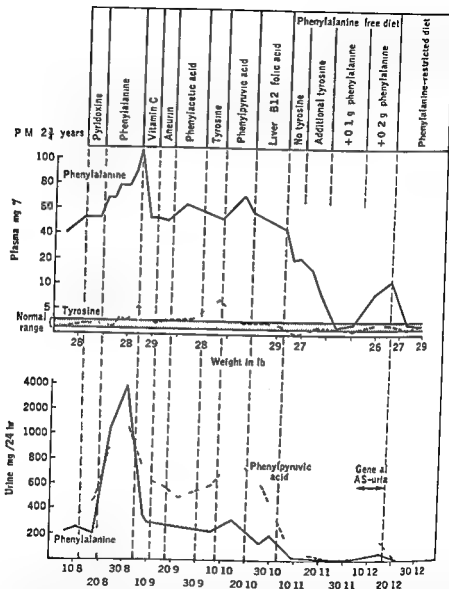


Fig 10-13 The biochemical response of a 3 year-old phenylketonuric girl to various substances and dietary regimens including a phenylalanine free diet. The latter reversed the biochemical abnormalities but later caused weight loss and general aminoaciduria (AS-uria) until enough phenylalanine for growth was restored (By permission of H. Bickel et al [53])

The abnormal components, which occurred in the  $\beta$  globulin fraction of plasma in about half the cases disappeared with treatment and reappeared when phenylalanine was restored to the diet [87]

### *Skin and Pigment Changes*

The eczema of those patients with this disturbance usually cleared promptly during treatment [69 67 168] only to recur if treatment was stopped. Hair (and possibly skin) pigmentation has increased with treatment in many individuals. This is best seen in the zone of new growth at the hair roots. The darkening can also be produced without lowering phenylalanine simply by increasing the tyrosine uptake to overcome the competitive inhibition of tyrosinase by phenylalanine.

### *Neurologic Signs*

Treatment ameliorates most of the neurologic signs of phenylketonuria with the exception of structural defects and those produced by severe brain damage [59]. A prompt effect on the behavior is usually observed. The patient becomes less restless and irritable and attention span increases [81]. An unmanageable child for example may develop appropriate fear of approaching automobiles. Motor performance improves and hyperactive reflexes hyperkinesia and tremors lessen. The muscular hypertonicity also decreases. When phenylalanine is administered the abnormalities promptly return and large doses intensify this effect.

### *Seizures and Electroencephalograms*

Severe and almost continuous seizures have been precipitated in the first few weeks of treatment in four cases [8 53 169 170]. Two additional cases with seizures and coma one ending fatally were associated with severe hypoglycemia. This developed after short fasting on the background of chronic undernutrition associated with low-calorie therapeutic diets [69]. The possibility of phenylalanine deficiency as the cause has been suggested.

The treatment almost always improves or normalizes the EEG and lessens or eliminates the seizures when continued for a sufficient period. The exceptions to this rule are those patients with the most severe neurologic impairment. Even in these patients conventional drug therapy may become more effective. Except for those with seizures as a complication developing early in treatment no seizures have developed for the first time during therapy. Treatment may affect favorably other neurologic signs within weeks but the full effect on the EEG and on seizure frequency is achieved only after months.

### *Intelligence*

The most important evidence that phenylalanine accumulation is the basic physiologic abnormality in phenylketonuria is the effect of phenyl-



TABLE 10-13 PHENYLKETONURIC PATIENTS TREATED WITH LOW PHENYLALANINE DIET WHEN UNDER 2 YEARS OF AGE

| C #   | S | Age treated          | Age last observed mo | Chemical | EEG as given                          | Dietary protein            | R for age |
|-------|---|----------------------|----------------------|----------|---------------------------------------|----------------------------|-----------|
| 3     | F | 10 d ya-             | 12                   | Good     |                                       | 100                        | [177]     |
| CM    | F | 2 wk-                | 3                    | Good     |                                       | N m l                      | [169]     |
| SR    | M | 3 wk-                | 11                   | Good     |                                       | III m l                    | [158]     |
| 1     | F | 4 wk-                | 34                   | Good     |                                       | GQ 88                      | [151]     |
| —     | F | 4 wk                 | 12                   | ?        | ?                                     | 94                         | [150]     |
| 2     | F | 6 wk-                | 28                   | Good     |                                       |                            | [173]     |
| CM    | M | 8 wk-                | 20                   | F        |                                       | IQ 90                      | [167]     |
| 1     | M | 8 wk-                | 42                   | Good     |                                       | DQ 100                     | [174]     |
| 8     | F | 16 wk-               | 16                   | G d      |                                       | GQ 64                      | [41]      |
| —*    | M | 17 wk-               | 30                   | Good     | ?                                     | IQ 100                     | [175]     |
| MY    | M | 19 wk-               | 11                   | Good     |                                       | III m l                    | [59]      |
| 1808  | F | 6 mo-                | 18                   | Fa       |                                       | DQ 85                      | [51]      |
| 190P  | M | 7 mo-                | 31                   | Good     |                                       | DQ 60                      | [51]      |
| R D   | M | 7½ 29 mo             | 42                   | Fa       | Sec m mal                             | IQ 63                      | [56]      |
| 1     | M | 9 mo-                | 15                   | F        |                                       | DQ 22                      | [174]     |
| MJ    | F | 10-15<br>( d 17 9m ) | 37                   | Good     | B ad mag<br>bn m l EEG<br>III dec eas | DQ 22                      | [59]      |
| —     | F | 12 mo-               | 18                   | Fa       | EEG abno m l<br>Site?                 | GQ IV                      | [71]      |
| J D   | F | 13 23 m              | 5½ (y)               | Fa       | EEG abno m l                          | DQ 97<br>High gr d<br>o R2 | [50]      |
| CM    | F | 15 mo-               | 24                   | Good     | Sec men m l                           | DQ 60-75                   | [58]      |
| 11    | M | 15 mo-               | 29                   | Fa       | EEG elight<br>imp em t                | GQ 32                      | [51]      |
| 2     | F | 15 mo-               | 23                   | F        |                                       | Id t                       | [176]     |
| 6*    | M | 16 mo-               | 38                   | G d      |                                       | GQ 68                      | [51]      |
| 6     | M | 16-19 mo             | 22                   | F poor   |                                       | IQ 49                      | [54]      |
| 19UP* | M | 18 mo-               | 2                    | F        | Imp o d EEG                           | IQ 28                      | [51]      |
| —     | M | 18 mo-               | 24                   | F poor   | Fat eaf q t                           | IQ 40                      | [57]      |
| 5     | F | 20 mo-               | 31                   | ?        | Fat ea d EEG<br>u b g d               | DQ 40                      | [171]     |
| 4     | F | 20 mo-               | 34                   | ?        |                                       | DQ 73                      | [171]     |
| 9     | F | 20 mo-               | 45                   | F        | EEG bn m l                            | DQ 31                      | [51]      |
| 10    | M | 20 mo-               | 6½ (y)               | Poo      | Ft o as d                             | GQ 34                      | [51]      |
| D W   | F | 22 35½ m             | 40                   | Good     | Sec men m l                           | (DQ) 45                    | [59]      |

N Comments EEG dose age (y) when b mites we a p r e t b e l t r e t m t Th n q t e t u s d t m e d i p m i a r g b l y m p b l e a f t h y d t e s t s / c h l s m o x 100 Th d g r e e f c h m e l o t r o l i s a e s t m e l m d a t a g d d d t h d l b a s e f t h m t l p h m p h y l l I I I l e o f < 0.5 (Good) < 10 (F) s d > 10 (Poo) p m l e s p e r m i d a g m o s t f t h p e r i o d. P h y l p y r u a c i d w l d b e t e d n t h P t l e d g r p C h a c m t d w t h a s t e r i c k ( ) w e t e s t e d w t h G o o d s o F a e t l d t e t e d b y d 2 y f g E l f t h e s e t e e c a c h d l p m t a l q u t a t 20 d a b

alanine deprivation on the intelligence of these patients. Reversal of intellectual impairment of older patients has not been realized but the low phenylalanine diet can prevent intellectual impairment during the first few years of life and may reverse recently established impairment [31 51, 55 59 171]

The effects of treatment are in complete accord with the view that the

phenylketonuric infant is normal at birth and that at about 6 months of age retardation begins and becomes progressively more severe. The results also indicate that this retardation is largely irreversible although apparently significant improvement has occurred in an occasional child over 3 years of age. There were no significant effects in a controlled study of the treatment of adults except that nonintellectual signs were ameliorated [31].

The dramatic effects of treatment are seen in patients diagnosed soon after birth and started on therapy during the first few months of life. Most of these have developed normally although the period of observation is still short. Gains appear to be maintained fairly well on cessation of therapy. Substantial improvement is also seen in children whose treatment is begun as late as 1 or 2 years of age.

All reported patients through 1958 whose treatment was started under the age of 2 years appear in Table 10-13. Inadequate biochemical control for shorter or longer periods occurred in many of the patients and some degree of damage was present in the older patients before treatment was started. These factors alone could explain the persistence of low developmental quotients in about half. Counting only those whose treatment was continued past 2 years of age and only the test results after this age which are less uncertain than earlier tests, about half the patients have developmental quotients of above 60. This is in striking contrast to the incidence of only 2 per cent of such high grade patients among untreated phenylketonuric patients. The elevation of over half of the early treated patients to the high grade group represents the most conservative estimate of the value and efficacy of the low phenylalanine dietary treatment of phenylketonuria.

## SUMMARY

1 The major biochemical changes in phenylketonuria can be traced through known chemical reactions to the accumulation in the tissues of that part of the dietary L-phenylalanine which would normally be converted to tyrosine. These changes are reversed when phenylalanine accumulation is avoided. The accumulation occurs because the specific liver enzyme phenylalanine hydroxylase is inactive.

2 The disease is inherited as an autosomal recessive character. The phenylalanine concentration of the serum of heterozygotes may be elevated but there are no definite deleterious phenotypic consequences of the heterozygous state.

3 The pathologic changes of skin and mind in phenylketonuria are also referable to the accumulation of phenylalanine since they are either reversed or prevented when the accumulation is avoided by a low phenylalanine diet. It is evident that treatment must be started early and



ketonuric defect since phenylalanine hydroxylase appears for the first time during the biochemical differentiation of the liver following birth. It is the failure to develop phenylalanine hydroxylase activity that is hereditary.

5 The consequences of the defect are best considered as the results of accumulation of (internal) environmental abnormalities which affect the tissues of the developing child. Some of these may be enzyme inhibitors and pharmacologic effects on preformed systems. Others may involve adaptive changes in the capacity of systems to handle unusual amounts of metabolites. All the effects should be and are found to be reversible, but those systems which still have to undergo biochemical differentiation when the chemical milieu becomes abnormal may be directed along an irreversible series of abnormal changes.

6 Too little is now known of the nature and control of embryologic and biochemical differentiation for understanding the intimate cellular consequences of the phenylketonuric abnormality, but when the chemical milieu becomes abnormal the brain is one of the few organs which must still differentiate biochemically, and it is irreversibly altered by this disease. The retardation of myelination is the only evidence besides functional tests that the brain is affected.

7 It is possible that late dentition and enamel hypoplasia also reflect the effects of the abnormal chemical milieu. Possibly other differentiations are altered, but the evidence is not convincing enough to include such items as hypogenitalism which are sometimes mentioned. Unspecified developmental abnormalities of a functional sort may contribute to the decreased survival of the affected patients. The outstanding problem in phenylketonuria is no longer the effect of the abnormal metabolism on the brain, but the nature of the alterations induced in the developing brain.

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continued at least until biochemical differentiation is complete. The step wise pathogenesis of the serious and permanent mental deficiency and other defects is less well understood than the biochemical disturbances. One of the abnormalities, melanin deficiency, is clearly referable

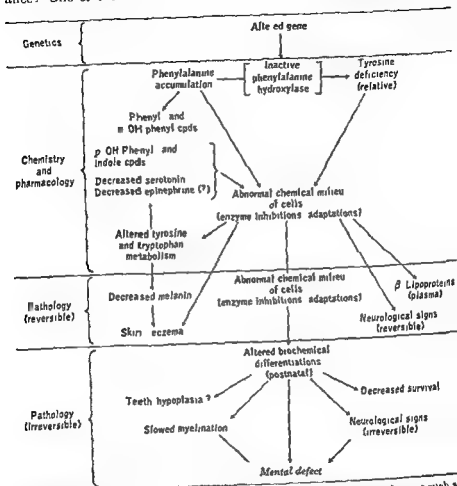


Fig 10-14 Scheme of pathogenesis in phenylketonuria. Irreversible changes such as the mental defect are considered to result from altered biochemical differentiation of the immature brain.

to competitive enzyme inhibition. Pharmacologic effects of the abnormal metabolites, especially derivatives of *o*-tyrosine and serotonin, may contribute to the symptomatology. The pathogenesis involving the chemical derangements and the reversible and irreversible pathologic changes is summarized in Fig 10-14.

4. The disease phenylketonuria, but not the primary defect, is acquired. Before and immediately after birth, all animals have the 'phenyl

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## Chapter 11

### Tyrosinosis

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Bert N. La Du

Classification of tyrosinosis as one of the inborn errors of metabolism is rather presumptive in view of the fact that only one case has ever been described and the inheritability of this condition has not yet been demonstrated. Nevertheless tyrosinosis has been generally accepted as a *bona fide* addition to the group of metabolic diseases associated with defects at particular steps in phenylalanine and tyrosine metabolism, i.e. albinism, phenylketonuria and alcaptonuria [1, 2].

In 1927 Medes, Berglund and Lohmann [3] found an unusual reducing substance in the urine of a patient with myasthenia gravis. The reducing compound was later isolated and identified as *p* hydroxyphenylpyruvic acid, the  $\alpha$  keto acid of tyrosine, and this condition was named *tyrosinosis* by Medes [4]. Other patients with myasthenia gravis were not found to excrete *p* hydroxyphenylpyruvic acid and no particular clinical symptoms were observed in the tyrosinosis patient which could be attributed to the excretion of the  $\alpha$  keto acid. Without doubt tyrosinosis must be a very rare disease. A search for additional cases made by Blatherwick among more than 20 000 persons with a weakly positive reduction test result for glucose in the urine failed to uncover any additional cases [5].

#### METABOLIC STUDIES IN TYROSINOSIS PATIENT

It is indeed fortunate that the unique case of tyrosinosis was in the hands of the capable investigator Grace Medes; her classic paper in 1932 [4] still remains the key source of information on this disease. The patient with tyrosinosis was a male Russian Jew, 49 years old at the time. Initial metabolic balance studies clearly showed that the excretion of *p* hydroxyphenylpyruvic acid and the other tyrosine metabolites increased proportionately to increases in dietary phenylalanine and tyrosine. The pattern of urinary metabolites found after placing the patient on various dietary and experimental feeding regimens is given in Table 11.1.

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It should be noted that *p*-hydroxyphenylpyruvic acid was excreted continually even during fasting or on a diet of very low tyrosine content. Under both these conditions about 1.6 gm of the  $\alpha$  keto acid was excreted per day. Medes pointed out that this is a reasonable figure for the amount expected from tyrosine derived from the catabolism of endogenous protein and she concluded that the metabolic defect was essentially complete. When the patient was placed on a regular diet the amount of urinary *p*-hydroxyphenylpyruvic acid doubled and tyrosine could then also be isolated from the urine. At still higher levels of tyrosine intake *p*-hydroxyphenyllactic acid was also excreted.

One of the most remarkable findings in the studies with this patient was the observation that feeding large amounts of tyrosine also led to the excretion of 3,4-dihydroxyphenylalanine (dopa). In fact the identification of dopa in the urine of this patient has been cited as one of the most convincing pieces of evidence that dopa is a normal metabolic product of phenylalanine and tyrosine in the higher animals [7]. It is assumed that in tyrosinosis the main pathway for tyrosine metabolism is blocked and therefore much more of this amino acid must be metabolized by alternative pathways than under normal conditions.

On the basis of the metabolites excreted in the urine of this patient and in order to emphasize the excretion of *p*-hydroxyphenylpyruvic acid perhaps a more appropriate name for this condition would be tyrosyluria [1]. One might refer to the case described by Medes as one of *essential* or *idiopathic tyrosyluria* to distinguish it from a number of other conditions in which tyrosyluria has been observed such as scurvy [8-10], pernicious anemia [11] and a variety of other diseases [12].

Spontaneous excretion of *p*-hydroxyphenylpyruvic acid was recently reported by Felix Leonhardt and Glaser [13] in two patients with liver disease and a previous splenectomy. In these patients, however, the amount of the  $\alpha$  keto acid excreted was regularly less than 50 mg per day and the amount exceeded this value only slightly when extra tyrosine was fed. Although the excretion of the  $\alpha$  keto acid in these patients is of considerable theoretic interest, the amount excreted is so much less than that found in the patient studied by Medes that it is more likely a different type of metabolic abnormality and therefore should not be classed as an additional case of tyrosinosis [1].

On the basis of the pattern of the urinary metabolites found in the urine of this patient, Medes proposed that the metabolic defect in tyrosinosis was a failure of the enzymatic step in which *p*-hydroxyphenylpyruvic acid is oxidized to homogentisic acid. It was believed that the excretion of *p*-hydroxyphenylpyruvic acid represented an inability to metabolize this  $\alpha$  keto acid and that as a consequence of this defect the earlier steps were also partially impaired, thus leading to the excretion of the closely related compounds when extra tyrosine was fed. The demon-



TABLE II 1 METABOLIC STUDIES WITH TYROSINOSIS PATIENT

| Diet and supplement  | Compounds excreted in the urine               |                 |  |  |
|--|---|-----------------|--|--|
|  | <i>p</i> -Hydroxyphenyl pyruvic acid gm/day   | Tyrosine gm/day | <i>p</i> -Hydroxyphenyl lactic acid gm/day | 3,4-Dihydroxyphenyl alanine                            |
| Fasting  | 1.54  | 0               | 0  | 0  |
| Tyrosine-free diet   | 1.64 1.43                                     | 0               | 0  | 0  |
| Mixed hospital diet  | 2.8   | 0.38            | 0  | 0  |
| High protein diet  | 3.2   | 0.80            | Trace                                      | 0  |
| High protein diet + 15 gm L-tyrosine   | 4.64  | 2.17            | 0.32                                       | + Urine turned black with alkali + 30 mg dopa isolated |
| High protein diet + 15 gm L-tyrosine   | 5.17  | 2.52            | 0.73                                       |  |
| Tyrosine-free diet + 2 gm <i>p</i> -hydroxyphenyl lactic acid                        | No increase                                   | 0               | 1.78 (4 days)                              | 0  |
| Tyrosine free diet + 4 gm <i>p</i> -hydroxyphenyl pyruvic acid on 2 consecutive days | Immediate increase continued for several days | 0               | Increase after 3 days for several days     | 0  |
| Tyrosine free diet + 2 gm 1-3,4-dihydroxyphenylalanine                               | Delayed increase maximum on 4th day           | 0.100†          | 0  | Nearly 0.800 gm recovered (incomplete)                 |

The unusual 2-day delay before an increase in *p*-hydroxyphenyl pyruvic acid was found and the rapid rate at which dopa is known to be metabolized [6] suggests that this increase may have been due to variation in the dietary intake of tyrosine rather than the test substance. It was suggested that the oxidation of tyrosine to dopa is reversible since some tyrosine was excreted after feeding dopa, but this is not supported by more recent studies [4]. Dehydroxylation of dopa to meta-tyrosine has been reported in rabbits [14] but not in man [7].

SOURCE: G. Medes [6].

can be increased by the administration of steroids [23] or by inducing stress conditions [18]

The next enzyme *p* hydroxyphenylpyruvic acid oxidase is the one proposed by Medes as defective in tyrosinosis. It catalyzes the oxidation

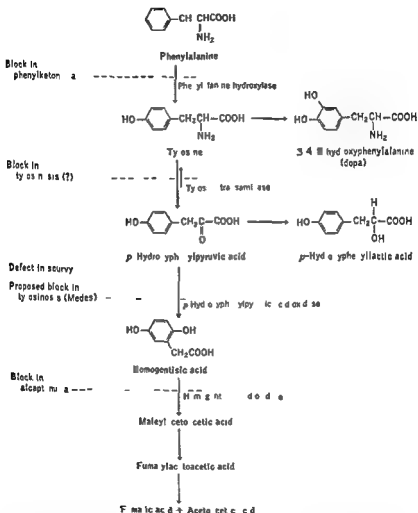


Fig 11.1 Metabolic blocks in the oxidation of phenylalanine and tyrosine

of *p* hydroxyphenylpyruvic acid to homogentisic acid and is present in the liver of various mammalian species including hog rabbit rat dog and man. It has also been detected though with lower activity in the kidney of some if not all of these species. Purified *p*-hydroxyphenyl

stration that administered homogentric acid was metabolized completely, even when given in relatively large amounts indicated that the defect must be located earlier in the pathway of tyrosine metabolism than it is in alcaptonuria (see Chap 12)

Even though the general scheme of tyrosine metabolism has undergone relatively little modification during the past 30 years the component enzymatic steps have been isolated and intensively investigated within this time Therefore, it is of interest to look again at the evidence upon which these interpretations were made 30 years ago and see whether in view of present understanding of the metabolic pathway the same conclusions would be reached today

## BIOCHEMICAL ASPECTS

Progress in understanding the metabolism of phenylalanine and tyrosine particularly during the past 10 years has been largely due to the successful isolation and purification of the enzymes catalyzing each of the reactions The enzymatic steps pertinent to this discussion are summarized in Fig 11-1 Since several excellent reviews have recently appeared which summarize the numerous papers concerned with these reactions [1, 8, 9, 15-17] only a few points will be mentioned here It is clear that most of the phenylalanine ingested is catabolized via its oxidation to tyrosine and that this hydroxylation step is essentially irreversible For this reason experiments carried out with administered phenylalanine should be equivalent to those in which tyrosine is given if the defect under study involves one of the steps in tyrosine metabolism

The main pathway for tyrosine metabolism in mammals is indicated in Fig 11-1 The first step a transamination reaction with  $\alpha$  ketoglutarate to yield *p* hydroxyphenylpyruvic acid appears to be the rate limiting step of the tyrosine oxidation pathway [18, 19] At present, it is believed that most of the *p*-hydroxyphenylpyruvic acid derived from tyrosine is formed via transamination and that relatively little of the  $\alpha$  keto acid results from oxidative deamination of the amino acid This transamination reaction is catalyzed by a specific transaminase which has been purified by Canellakis and Cohen [20, 21] from dog liver and more recently by Kenney [22] from rat liver L-tyrosine phosphate is the coenzyme and neither pyruvate nor oxaloacetate can replace the requirement for  $\alpha$  ketoglutarate The enzyme is specific for L-tyrosine in that no activity is found with phenylalanine tryptophan histidine or a number of other amino acids The transaminase is strongly inhibited by sulfhydryl reagents such as iodoacetate and *p* chloromercuriphenyl sulfonate but it is not inhibited by metal binding agents such as  $\alpha$  a dipyridyl diethyldithiocarbamate and 8 hydroxyquinoline Recently evidence has been obtained that the transaminase activity in rat liver

most of the tyrosine is degraded via *p*-hydroxyphenylpyruvic acid to homogentisic acid

## METABOLIC DEFECT IN TYROSINOSIS

Medes proposed [4] on the basis of the tyrosine metabolites found in the urine that the defect in tyrosinosis must be the lack of *p*-hydroxyphenylpyruvic acid oxidase activity. The constant excretion of the  $\alpha$ -keto acid accompanied by closely related compounds when tyrosine was fed suggested that the primary difficulty was in metabolizing this acid and that as a consequence the preceding steps were secondarily impaired.

It is of interest to reexamine the balance study data from the tyrosinosis patient in view of current understanding of tyrosine metabolism and to speculate as to whether the same conclusions would be drawn today. There is no reason to doubt that the metabolic defect occurs early in the scheme of tyrosine metabolism. There are however several observations not in accord with the localization of the defect at the *p*-hydroxyphenylpyruvic acid oxidase step. These are the finding that the predominant metabolite was *p*-hydroxyphenylpyruvic acid with very little *p*-hydroxyphenyllactic acid, the apparent completeness of the block at low levels of tyrosine intake yet its incompleteness at moderate or high levels of tyrosine intake and the excretion of dopa when tyrosine was fed.

There is a striking difference in the tyrosyluria found in the patient with tyrosinosis from that present in other conditions in which the excretion of *p*-hydroxyphenylpyruvic acid has been observed. In tyrosinosis only *p*-hydroxyphenylpyruvic acid was excreted (except on a diet high in tyrosine) whereas in the other conditions *p*-hydroxyphenyllactic acid was a major tyrosyl product in the urine. For example premature infants fed extra tyrosine [10] were found to excrete several times as much *p*-hydroxyphenyllactic acid as *p*-hydroxyphenylpyruvic acid. Scorbatic guinea pigs [35] and children [36] also excrete appreciable quantities of the lactic derivative. This difference in the tyrosyluria was not due to an inability of the tyrosinosis patient to reduce the  $\alpha$ -keto acid since he excreted a small amount of *p*-hydroxyphenyllactic acid when the diet contained large amounts of tyrosine (Table 11.1). Furthermore when the patient was given *p*-hydroxyphenylpyruvic acid a considerable portion of it was excreted as *p*-hydroxyphenyllactic acid. The latter acid was isolated from the urine and found to be optically active and it

The suggestion has been made [9, 4] that the patient with tyrosinosis may have had unrecognized vitamin C deficiency but the difference in the *p*-hydroxyphenyllacturia in this condition from that found in scorbatic individuals would seem to exclude this possibility.

pyruvic acid oxidase has been prepared from beef [24] pig [24, 25] and dog liver [26-28], and properties of the purified enzyme have been described

The conversion of *p* hydroxyphenylpyruvic acid to homogentisic acid requires two atoms of oxygen and the liberation of one molecule of CO<sub>2</sub>. The reaction is complex, it involves hydroxylation of the aromatic ring migration of the side chain, and oxidation and decarboxylation of the pyruvate to an acetate side chain. Although it has long been considered probable that 2,5 dihydroxyphenylpyruvic acid is an intermediate in this oxidation recent studies with *p* hydroxyphenylpyruvic acid oxidase preparations from several animal sources [24-26] have shown that 2,5-dihydroxyphenylpyruvic acid is not oxidized to homogentisic acid. Furthermore incubation of 2,5 dihydroxyphenylpyruvic acid with normal human liver homogenate (or liver homogenate from an alcaptonuric person) does not yield homogentisic acid [19]. The observation made by Neubauer [29] that feeding 2,5-dihydroxyphenylpyruvic acid led to extra homogentisic acid production in an alcaptonuric patient is not inconsistent with these observations if one assumes that even though free 2,5 dihydroxyphenylpyruvic acid is not an intermediate in tyrosine metabolism this compound may be converted to homogentisic acid by some other mechanism.

*p* Hydroxyphenylpyruvic acid oxidase is highly specific for its substrate and does not catalyze the oxidation of other hydroxyphenylpyruvic acids or any of a number of related compounds [24-26]. The enzyme requires either ascorbic acid or one of the group of compounds which can replace the vitamin such as the reduced form of 2,6-dichlorophenol indophenol in vitro [15]. These agents are required in considerably less than stoichiometric amounts and appear to function by preventing a gradual inhibition of the enzyme by its substrate which would occur in the absence of the protective agent [26-28]. Ascorbic acid seems to act in this protective capacity in vivo [30]. Although it was proposed several years ago that ascorbic acid acted as a coenzyme for *p*-hydroxyphenyl pyruvic acid oxidase [31-32] its function in this reaction now seems to be much less specific [15, 33, 34] and unlike the coenzyme functions associated with the members of the B vitamins.

The enzyme can be inhibited by relatively low concentrations of diethyldithiocarbamate [26] and by sulfhydryl binding agents such as *p*-chloromercuribenzoic acid [24]. In view of the known affinity of diethyldithiocarbamate for copper a search has been made for this metal in the purified enzyme but there is no evidence that it is a copper containing protein [24, 26].

The occurrence of homogentisic acid as a normal metabolite was subject to some dispute in the past but there is now general agreement that

tyrosine but not of *p*-hydroxyphenylpyruvic acid. The  $\alpha$ -keto acid rather than tyrosine or *p*-hydroxyphenyllactic acid would be the expected main metabolite in the urine just as was found. In addition an elevated tissue level of tyrosine would be more likely to lead to extra dopa being synthesized than would a block in the oxidation of *p*-hydroxyphenylpyruvic acid. If the keto acid which was excreted arose by the metabolism of tyrosine within the kidney (analogous to the keto acid from phenylalanine in phenylketonuria) it would not be surprising to find this mechanism saturated at moderate levels of tyrosine intake. This is in accord with the apparent incompleteness of the block when high dosages of tyrosine were given to the patient.

If another patient with tyrosinosis should ever be found it would be particularly important to determine the plasma levels of tyrosine and *p*-hydroxyphenylpyruvic acid. Fortunately a number of microenzymatic methods are now available for these determinations [42-49]. The ultimate proof that a particular enzyme is missing in tyrosinosis will of course depend upon the availability of suitable tissues from a patient with this disease.

### SUMMARY

1 Tyrosinosis is a rare metabolic disease of tyrosine metabolism characterized by the excretion of *p*-hydroxyphenylpyruvic acid. Other tyrosyl metabolites are also excreted when large amounts of this amino acid are given. Only one case of the disease has ever been found and the inheritance of the condition has not been demonstrated.

2 It is generally assumed that the enzyme system defective in tyrosinosis is *p*-hydroxyphenylpyruvic acid oxidase. However the defect may well be in the preceding step, tyrosine transaminase. Evidence supporting the localization of the defect in each of these steps is discussed.

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was presumed to arise by the enzymatic reduction of *p* hydroxyphenyl pyruvic acid. Meister [37] has since reported that *p* hydroxyphenyl pyruvic acid is active as a substrate with the lactic acid dehydrogenase of muscle.

On the basis of these results one would expect that if *p* hydroxyphenyl pyruvic acid were circulating in the tissues of the tyrosinosis patient much more reduction and excretion of the lactic acid analogue would take place. It is difficult to understand why only the  $\alpha$  keto acid was excreted if the defect were the lack of liver *p* hydroxyphenylpyruvic acid oxidase activity.

On the other hand the urinary metabolites found in the urine in premature infants and in scorbutic guinea pigs and man are those one would expect to find with an impaired activity of liver *p* hydroxyphenyl pyruvic acid oxidase. Evidence that this enzyme is relatively deficient in premature infants has been obtained by Kretchmer et al [38-39]. It has also been recently shown that about one half of liver *p* hydroxyphenylpyruvic acid oxidase activity is inhibited by the administration of *p* hydroxyphenylpyruvic acid to scorbutic guinea pigs. Under these conditions the activities of tyrosine transaminase and homogentisic acid oxidase are not affected [30].

Attempts have been made to induce experimental tyrosinosis in guinea pigs by inhibiting *p*-hydroxyphenylpyruvic acid oxidase with diethyl dithiocarbamate [40]. The administration of this compound results in almost complete inhibition of the oxidase *in vivo*. Animals so treated were injected with *p* hydroxyphenylpyruvic acid intraperitoneally and even under these conditions very little of the  $\alpha$  keto acid was excreted unchanged. The main tyrosyl product in the urine was *p* hydroxyphenyllactic acid [41].

It appears therefore that in the various conditions in which liver *p* hydroxyphenylpyruvic acid oxidase is known to be impaired one finds a relatively large amount of *p* hydroxyphenyllactic acid in addition to *p* hydroxyphenylpyruvic acid in the urine.

In view of these observations the different pattern of tyrosyluria found in the patient with tyrosinosis raises the possibility that this metabolic disorder may not be due to a defect in *p* hydroxyphenylpyruvic acid oxidase. It also seems unlikely that *p* hydroxyphenylpyruvic acid was circulating in the tissues except when the intake of tyrosine was very high since the ability to reduce the  $\alpha$  keto acid to *p* hydroxyphenyllactic acid was demonstrated to be present in this patient (Table 11 I).

One possibility is that the metabolic error in tyrosinosis is much like that found in phenylketonuria with the primary defect a failure to metabolize the amino acid even though the corresponding  $\alpha$  keto acid is the main abnormal urinary metabolite. If tyrosinosis were analogous to phenylketonuria one would expect to find a high level of circulating

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Boedeker precipitated homogentisic acid from alcaptonuric urine as the lead salt [2-3] but he was unable to obtain enough material to purify it and determine its exact chemical structure. He did point out the similarity of its behavior in alkali with that of known hydroxyphenols.

During the following 30 years there was considerable confusion as to the chemical structure of "alkapton." It was variously reported by different groups to be catechol [4-5], protocatechuic acid [6], "glycosuric acid" [7] and uroleucic acid [8]. The various claims and disputes about its composition during this period are well summarized in a recent review by Knox [9]. The controversy ended and the chemical structure of alcapton was firmly established in 1891 by the excellent work of Wolkow and Baumann [10]. They identified it as 2,5-dihydroxyphenyl acetic acid and named it *homogentisic acid* because of its close structural relationship to gentisic acid (2,5-dihydroxybenzoic acid). The earlier reports of other substances such as uroleucic acid in alcaptonuric urine were shown to be the result of analytical errors from contamination with various normal urinary constituents [11].

Once the aromatic structure of homogentisic acid was known, it was not long before various suggestions were made as to the source of this unusual urinary product. The known aromatic substances in proteins, tyrosine and phenylalanine, were of course the primary suspects (Fig 12-1). Wolkow and Baumann demonstrated in 1891 [10] that feeding

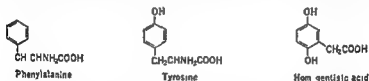


Fig 12-1 Formulas of phenylalanine, tyrosine, and homogentisic acid

either extra tyrosine or a diet high in protein greatly increased the amount of homogentisic acid excreted by an alcaptonuric patient. Even though they were incorrect in believing that the formation of homogentisic acid was due to the activity of bacteria in the gut, their important observations initiated a number of studies by clinical investigators of that time who used the metabolic defect in alcaptonuric individuals to determine the pathway by which phenylalanine and tyrosine are metabolized to homogentisic acid. Numerous compounds which were possible

alkali, and the Greek root *αλκω* meaning to seize, to possess, the combination therefore meaning "to have an affinity for alkali." *The Oxford English Dictionary* [165] gives the correct origin and indicates that the *k* of alcaptonuria comes from the Greek root *αλκω* rather than from alkali. Transliteration of the *k* to *c* in the prefix follows the same pattern used in many words derived from similar Greek roots.

## Chapter 12

### Alcaptonuria

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Bert N. La Du

Alcaptonuria is a rare hereditary metabolic disease in which the enzyme homogentisic acid oxidase is missing. Because of this defect, homogentisic acid produced during the metabolism of phenylalanine and tyrosine cannot be further metabolized; it therefore accumulates and is excreted in the urine.

If urine containing homogentisic acid is allowed to stand for some time, it gradually turns dark as the acid is oxidized to a melanin-like product. The polymerization is speeded by alkali; this explains why washing diapers of alcaptonuric infants with soap tends to make the stains more intense instead of removing them.

It is not surprising that such an obvious sign as dark urine led to the early recognition of this disease. Several persons reported in the medical literature of the sixteenth and seventeenth centuries who continually passed dark urine are presumed to have had alcaptonuria—see Garrod [1]. The first patient in whom the diagnosis was made with certainty was one described by Boedeker in 1859 [2]. He recognized that the reducing properties of this patient's urine were different from those of one containing glucose (for example, it did not reduce bismuth hydroxide) and he observed the darkening of the urine when alkali was added. He used the property of avid oxygen uptake in alkaline solution to give the substance a name: *'in alkalischer Lösung bei gewöhnlicher Temperatur den Sauerstoff begierig zu verschlucken und nannte ihn danach Alkapton (freilich recht barbarisch zusammengesetzt aus dem arabischen alkali und dem griechischen καρτελ begierig verschlucken)'* —[2 p. 139]. Two years later [3] Boedeker spelled it 'Alkapton' and since then this condition has been known as *Alkaptonurie* in the German literature and as *alcaptonurie* in the French. Both *c* and *k* have been used by writers in English.<sup>1</sup>

<sup>1</sup> Several medical dictionaries erroneously assume alkapton to be derived from

metabolism in 1909 [12] the first such scheme for any of the amino acids. In 1928 [13] he revised it to incorporate the results obtained during the intervening years (Fig 12.2). Although a few changes have been made since then the basic scheme which he postulated has remained essentially unchanged during the last 30 years.

Studies on alcaptonuria have been of general importance in the development of ideas about diseases of metabolism. As a result of his studies on alcaptonuria Sir Archibald Garrod developed his whole concept of inheritable metabolic diseases. In 1908 he discussed alcaptonuria in one of the Croonian Lectures [1] and in the following year he expanded his ideas more completely in his classic book *Inborn Errors of Metabolism* [11]. He thought of alcaptonuria as a metabolic "freak" or "sport" comparable to a structural abnormality rather than as a disease in the usual sense. He felt that patterns of metabolism varied in each individual according to his hereditary background and that alcaptonuria and the other inborn errors of metabolism represented extreme examples of such variant possibilities [14]. He suspected that these variations ultimately might depend upon differences in the activity of specific enzymes thus anticipating by many years the conclusion of Beadle and Tatum [15] that a single defective gene is correlated with a metabolic block in one enzymatic reaction.

In 1909 Garrod [11] wrote of the probable defect in alcaptonuria:

We may further conceive that the splitting of the benzene ring in normal metabolism is the work of a special enzyme that in congenital alcaptonuria this enzyme is wanting whilst in disease its working may be partially or even completely inhibited. The experiments of G. F. Imbden and others upon perfusion of the liver suggest that organ as the most probable seat of the change.

Garrod's supposition that a specific enzyme is missing in alcaptonuria has been supported through the years by many types of circumstantial evidence and recently has been confirmed by direct biochemical assay of alcaptonuric liver preparations [16].

## CLINICAL FEATURES

The cardinal features of alcaptonuria are signs due to the presence of homogentisic acid in the urine: pigmentation of cartilage and other connective tissues and nearly always in later years arthritis.

### URINARY CHANGES

According to the usual textbook description people with alcaptonuria give a history of dark urine or urine which turns dark on standing. It should be emphasized that in a large number of alcaptonuric patients this finding is not observed. Many patients have never noted any ab-

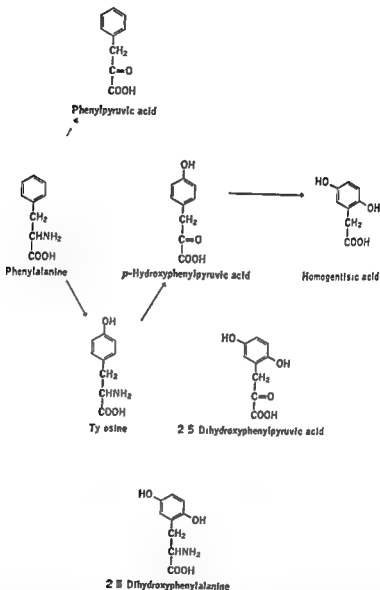


Fig 172 Scheme of phenylalanine and tyrosine metabolism to homogentisic acid based upon feeding experiments with alcaptonuric patients [15]. The dotted arrows show various pathways considered possible by Neubauer; the solid arrows indicate the pathway as it is viewed today.

intermediary substances in the formation of homogentisic acid were fed to alcaptonuric patients. It was expected that compounds in the metabolic sequence would increase the excretion of homogentisic acid but that those which were not intermediates would fail to do so. On the basis of such studies Neubauer suggested a preliminary scheme of tyrosine

isolated from the urine after precipitation as the lead salt [27, 31] and the product shown to have the correct chemical composition and melting point. Paper chromatography of the urine directly or of the product obtained by extracting acidified urine with ether, furnishes a simple technique to identify homogentisic acid [32]. More recently a specific enzymatic method has been developed which permits the quantitative analysis of homogentisic acid in urine, blood and other tissues [33, 34].

### OCHRONOSIS

In 1866 Virchow described a peculiar type of generalized pigmentation in the connective tissues of a 67 year-old man [35]. The pigment was gray to bluish black grossly but an ochre color microscopically and for this reason he named the condition *ochronosis*. Although the patient's clinical history is not known it is quite certain that Virchow described for the first time the generalized pigmentation which gradually develops in alcaptonuria. Actually it was not until nearly 40 years later that Albrecht in 1902 [36] clearly demonstrated the connection between ochronosis and alcaptonuria. Not long after Osler diagnosed ochronosis clinically for the first time in two alcaptonuric brothers [37]. He recognized that the pigmentation of the sclerae and ears were signs of the same metabolic abnormality that had previously been detected only by changes in the urine. Perhaps it is not unexpected that there was such a delay in the clinical recognition of the ochronotic pigmentation in alcaptonuria. Generally the earliest change that can be detected externally is a slight pigmentation of the sclerae or the ears but these changes are rarely noticeable before the alcaptonuric patient is 20 or 30 years old. The eye pigmentation is usually found about midway between the cornea and outer and inner canthi at the site of the insertions of the recti muscles (Fig. 12-3). In addition a more diffuse pigmentation may also involve the conjunctiva and cornea [39]. The typical pigmentary changes in the ear cartilages similarly occur only in long-standing alcaptonuria. The cartilage is slate blue or gray and feels irregular and thickened. It is first seen in the concha and the antihelix and later in the tragus. It is sometimes reported that a dusky discoloration corresponding to the underlying tendons can be seen through the skin over the hands. The prominence of this pigmentation is variable and in many instances it is scarcely evident at all. The pigment appears in perspiration clothing near the axillary regions may be stained and the skin may have a brownish discoloration in the axillary and genital regions [24].

In contrast to these rather minimal findings the pigmentation observed in the tissues of an elderly alcaptonuric patient at operation or at post mortem is indeed striking [30, 40-42]. Cartilage in many areas particularly the costal laryngeal and tracheal cartilage is densely pigmented and is described as being coal black in some areas. Pigmentation

normality in the color of the urine during childhood [17-19] and diagnosis has been made only after they sought treatment for arthritis during their later years [20-23]. In some cases diagnosis has followed a false-positive test for diabetes [20, 22] or the finding of the unusual and distinctive x-ray changes in the spine [24]. In others the disease has not been suspected until a surgical procedure has revealed marked pigmentation of the cartilage [25].

Alcaptonuric individuals on a normal diet void a urine which at first is not an abnormal color and which does not darken for many hours if it remains at an acid pH. This is true even for patients with extensive ochronosis. It appears therefore that in those instances where freshly voided urine turns dark quickly additional factors must be involved. Two factors that would favor rapid darkening are the excretion of an alkaline urine and a lower concentration than normal of vitamin C and possibly other reducing agents usually present in the urine. It is well known that vitamin C protects homogentisic acid against oxidation and in the past vitamin C has been suggested as a therapeutic agent because of this property [26].

The unusual findings in alcaptonuric urine can all be attributed to one abnormal constituent homogentisic acid. No abnormal amino acid pattern [17] or other tyrosine metabolic products are found [27].<sup>2</sup> The various diagnostic tests for alcaptonuria by urinalysis; therefore, are all based upon the detection of homogentisic acid through its unusual chemical properties. Its ease of oxidation results in a gradual darkening of the urine downward from the surface until the entire sample is dark brown; this darkening is greatly accelerated by alkali. Further evidence of its ease of oxidation is the behavior of alcaptonuric urine in its reaction with Benedict's sugar reagent. Homogentisic acid not only reduces the copper reagent to yield a yellow-orange precipitate but it also undergoes darkening because of the alkalinity of the reagent. The net effect is an orange precipitate in a muddy brown solution. The reduction of molybdate is the basis of the Brigg's test commonly used to follow the urinary excretion of homogentisic acid [28]. Reduction of silver in photographic paper emulsion has been used as a qualitative test [29] and as the basis of a quantitative method to measure this acid [30]. Homogentisic acid is not fermented by yeast and it does not fluoresce under ultraviolet light.

A presumptive diagnosis of alcaptonuria can be made on the basis of the results of these nonspecific tests but a more specific means for its identification is desirable. In many cases homogentisic acid has been

<sup>2</sup> It has been reported that an alcaptonuric patient excreting about 7 gm homogentisic acid also excreted about 0.5 mg gentisic acid per day [166]. The conversion of small amounts of homogentisic acid to gentisic acid has been demonstrated in homogenates of rabbit liver [167].

been determined. It is possible that some other constituents in addition to homogentisic acid are included in the product just as melanin obviously contains more than a polymerized dopa unit i.e., a considerable quantity of sulfur [45].

The formation of the pigment in the tissues may be entirely non enzymatic like the darkening of alcaptonuric urine. Solutions of pure

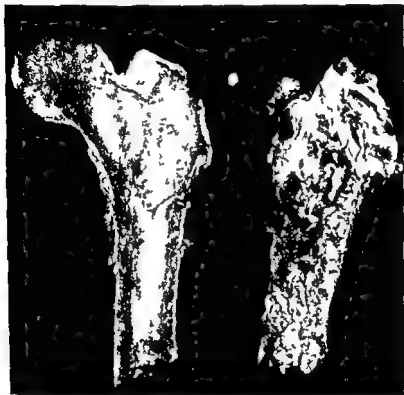


Fig. 12-4. Ochronotic pigmentation of the femur of a 56-year-old alcaptonuric subject (Courtesy of Dr. H. W. Edmonds of the Washington, Hospital Center Washington D.C.)

homogentisic acid made alkaline and aerated with air or oxygen form a dark brown product which has an ultraviolet absorption peak at 250 m $\mu$ —Milch et al [46]. Unfortunately the pigment deposited in the tissues of an alcaptonuric patient with ochronosis has not been analyzed in the same way and so the relevance of the model polymerization to the *in vivo* process has not been established. Milch and Titus [47] have recently suggested that since polymerization of pure homogentisic acid solutions does not proceed at neutral pH and low partial pressure of oxygen (as occurs in cartilage) it is likely that if molecular oxygen is



is also present throughout the body in fibrous tissues, fibrocartilage tendons and ligaments (Fig 12-4). To a lesser degree it is also found in the endocardium, the intima of the larger vessels in various organs such as kidney and lung and in the epidermis. Microscopic examination shows the pigment to be deposited both intercellularly and intracellularly, and it may be either granular or homogeneous. Like melanin, the ochronotic pigment is bleached when treated for 24 hr with hydrogen peroxide and it is soluble in alkali, but only slightly soluble in hydrochloric acid.



Fig. 12-3 The bilateral deposition of ochronotic pigment in the sclerae best seen in the left eye [33].

Thus in many of its chemical characteristics the ochronotic pigment resembles melanin arising from 3,4-dihydroxyphenylalanine (dopa). Unfortunately, there is no specific stain to distinguish the ochronotic pigment of alcaptonuria from melanin derived from other sources. Although Fitzpatrick [43] states that Becker's silver stain for melanin is not darkened by ochronotic pigment and that the latter is stained intensely black with polychrome methylene blue, more recently Cooper and Moran [42] compared the staining properties of ochronotic pigment and melanin employing a number of special stains. They concluded that no specific differentiation can be made with any of the stains used. Both pigments were best detected by the Nile blue stains of Lillie [44]. Variations with trichrome, cresyl violet and the periodic acid-Schiff (PAS) stains seemed to depend mainly upon the differences in the amount of pigment present rather than the type of pigment represented.

The pigment deposited in ochronosis is presumably a polymer derived from homogentisic acid, but its exact chemical structure has not yet

culosis. No mention was made of ochronotic changes in their connective tissues but it is unlikely that if they were present to the degree found in most alcaptonuric patients they would have gone unnoticed.

### ARTHRITIS

'Ochronotic arthritis' is a manifestation of long standing alcaptonuria. From the case reports in the literature <sup>11</sup> it appears that alcaptonuric arthritis occurs at an earlier age and is more severe in males than in

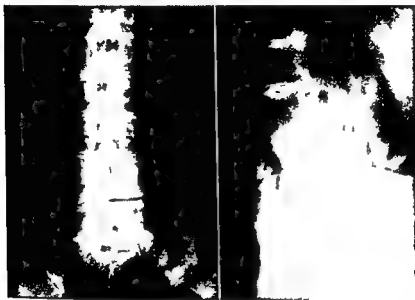


Fig 12.5 Ro ntgenograms of the spine showing the typical narrowing and calcification of the intervertebral disks [58]

females [61] even though the sex incidence of alcaptonuria is roughly equal. This sex difference in ochronotic arthritis is reminiscent of the similar preponderance of gouty arthritis in males. Hench [62] has stated that ochronotic arthritis resembles rheumatoid arthritis clinically but osteoarthritis roentgenographically. The earliest symptoms observed are usually some degree of limitation of motion of the hip, knee joints or occasionally the shoulders. There are nearly always periods of acute inflammation which may resemble rheumatoid arthritis and later there is usually rather marked limitation of motion and ankylosis in the lumbosacral region.

X-rays may reveal changes considered almost pathognomonic of alcaptonuria [24]. The vertebral bodies of the lumbar spine show degeneration of the intervertebral disks with a narrowing of the space and dense calcification of the remaining disk material (Fig 12.5). This is

involved in *vivo* it involves the participation of an enzyme system present locally. This conclusion assumes not only that the composition and steps of formation of the ochronotic pigment would be the same in *vivo* as in solutions of pure homogentisic acid, but that the pigment is both formed and deposited within the cartilage. It is not possible today to say to what degree enzymes play a role in the synthesis of the ochronotic pigmentation in alcaptonuria although on the basis of their specificity it seems reasonable to exclude the enzymes involved in the synthesis of melanin via dopa.

#### OCHRONOSIS NOT DUE TO HOMOGENTISIC ACID

Clinically ochronosis due to alcaptonuria might possibly be confused with the pigmentation of the skin, nail beds, conjunctivas and cartilage seen in persons who have taken Atabrine for many months [48]. Of course the history and the failure to find homogentisic acid in the urine should establish the diagnosis.

Another type of acquired ochronosis is that which is secondary to the prolonged use of carbolic acid dressings for chronic cutaneous ulcers [49-54]. This pigmentation is reversible and recedes after the medication is discontinued. Since this agent is rarely used today, nearly all the cases of ochronosis seen now are secondary to alcaptonuria. There are however a few puzzling cases of ochronosis in the literature in which exogenous agents such as phenol can probably safely be excluded as causative agents and in which the urine is reported to contain no homogentisic acid [55-56]. In the case described by Oppenheimer and Kline [55] a determined effort to isolate homogentisic acid from the urine was made by Janney [57]. He concluded that this was an example of ochronosis secondary to a melanuria but no aromatic metabolite related to dopa was identified in the urine. He was able to isolate a melanin like pigment from the urine and from a prostatic calculus which had by elementary analysis much the same values as those reported by Morner [46] for melanin obtained from the urine and tumor tissue of a patient with a melanosarcoma. It is possible that melanotic tumors might cause a generalized ochronosis also but it is unlikely that a patient with such a condition would survive long enough for this complication to become evident [50].\*

On the other hand instances in which alcaptonuria unaccompanied by ochronosis has been diagnosed with certainty are extremely rare. From the nature and completeness of the metabolic defect one would expect that all alcaptonuric patients would develop ochronosis to some degree if they live to middle age. The two patients with alcaptonuria who did not have ochronosis at autopsy [59-60] had extensive tuber

\* In one case of alcaptonuria the ochronotic pigment in the eye was misdiagnosed as a melanosarcoma and the eye was removed [58].

nosis and cardiovascular disease is not clearly established but a review of the case histories of alcaptonuric patients indicates that there is a high incidence of heart disease [22]. In 1910 Beddard [52] tabulated the autopsy findings in 11 cases of ochronosis (none due to treatment with phenol) and found that 8 had chronic mitral and aortic valvulitis, 1 had an aortic aneurysm and 1 an aneurysm of the left ventricle. Other investigators [40-65] have noted generalized arteriosclerosis and calcification in the heart valves and of the annulus of the aortic and mitral valves [41]. Myocardial infarction is a common cause of death in this group.

Other complications reported in alcaptonuric patients are ruptured intervertebral disks [66] and prostatitis [17, 22, 55, 67] or renal stones—see Young [67]. Clinical case reports on new cases of alcaptonuria should be encouraged in order that additional phenomena related to this metabolic disease may be revealed. One case of alcaptonuria with polycythemia [68] and one with severe renal disease called "ochronotic nephrosis" [42] remain isolated examples presumably because of chance association with other diseases. It should be kept in mind that conditions which favor the expression of alcaptonuria, such as consanguineous marriages, would also favor the manifestation of other recessive but unrelated traits.

## SYNTHESIS AND DEGRADATION OF HOMOGENTISIC ACID

### BIOSYNTHESIS

In mammals most of the dietary phenylalanine and tyrosine is oxidized to acetoacetic acid by enzyme systems localized primarily in the liver and kidney. The scheme of this metabolic pathway is shown in Fig. 12-6. Several excellent reviews of phenylalanine and tyrosine metabolism have appeared during the past few years [69-72]. These may be consulted for the detailed experimental evidence supporting each of the steps in this scheme. As mentioned above, the scheme is based upon the earlier studies with alcaptonuric patients and many animal experiments *in vivo*. It has been revised and extended, particularly during the past 10 years, by a large number of experiments on tyrosine metabolism *in vitro*.

In some of these *in vitro* studies isotopically labeled phenylalanine and tyrosine were used to determine the fate of each of the carbon atoms in the aromatic ring and of the side chain [73-77]. The labeled amino acids were incubated with liver slices and the distribution of the isotope was determined in the products CO<sub>2</sub>, acetoacetic acid, and fumaric acid. The experiments showed that two of the four carbon atoms of acetoacetic acid were derived from carbon atoms 2 and 3 of the side chain and that the other two came from the ring. Furthermore, the position of the isotope from the ring carbon atoms indicated that the side chain must have migrated during the oxidation (Fig. 12-7). The isotopic evidence

accompanied to a variable degree by fusion of the vertebral bodies. From the x ray changes in the lumbar spine alone, it is often possible to be reasonably certain of the diagnosis of alcaptonuria. In contrast to rheumatoid spondylitis little osteophytic formation and minimal calcification of the intervertebral ligaments are present. The large peripheral joints involved also differ from osteoarthritis in that the degenerative joint changes in ochronotic arthritis are most commonly the shoulder and hip whereas such joints as the sacroiliac may be completely spared.

Calcification of the ear cartilage is another sign of the disease that may be observed by x ray. The large joints affected generally show degenerative osteoarthritic changes with calcified deposits most commonly in the muscle tendons around the large joints. Occasionally free intraarticular bodies are found [63]. By contrast, the smaller joints usually show little or no abnormality.

The common occurrence of arthritis in the general population and the long period before its onset in patients with alcaptonuria no doubt account for the failure of the earlier investigators to appreciate the association of arthritis with alcaptonuria. The first case described by Boedeker was reported to have neuralgia of the lower lumbar spine. The early investigators considered alcaptonuria a completely benign disease without symptoms and of clinical importance only in that it might be misdiagnosed as diabetes. A review of the earlier case reports shows that in most instances osteoarthritis was mentioned and indeed, nearly all alcaptonuric patients develop arthritis during their later years. The arthritic complications are often severe and painful and may lead to a completely bedridden existence in later life.

The relationship between the deposition of pigment in the connective tissue and the degenerative changes which occur in some areas of the connective tissues particularly the cartilage and the intervertebral disks remains unknown. It has been proposed that the pigment acts as a chemical irritant to accelerate a degenerative process in the cartilage leading to changes similar to those in osteoarthritis [21, 63]. There is no evidence to support this suggestion. It is also possible that either the pigment or homogentisic acid itself might inhibit some of the enzyme systems involved in cartilage metabolism. Recently Greiling [64] has shown that low concentrations of the pigment prepared by treating homogentisic acid with alkali inhibit the action of hyaluronidase on chondroitin sulfonic acid and on hyaluronic acid but homogentisic acid does not act as an inhibitor at the same concentrations.

#### OTHER FINDINGS IN ALCAPTONURIA

In addition to the features mentioned above other complications seem to occur in alcaptonuric patients with a greater frequency than might be anticipated in the general population. The relationship between ochro-

was entirely in agreement with the scheme postulated earlier by Neubauer [13] (Fig 12 2) to account for the 2,5-dihydroxyphenyl intermediary product homogentisic acid from the 4-hydroxyphenyl substrates. This rearrangement was believed to involve a quinol intermediate with a migration of the side chain much like the migration of the methyl group in the oxidation of *p*-cresol [78] (Fig 12 8). This unusual migration in the

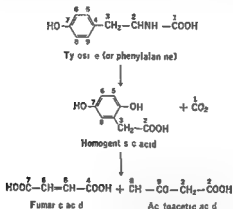


Fig 12 7 Fate of each of the carbon atoms of phenylalanine or tyrosine based upon experiments with the amino acids labeled with isotopic carbon

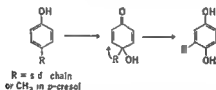


Fig 12 8 Formation of quinol intermediate and migration of the substituent (CH<sub>3</sub> in *p*-cresol or side chain) in *p*-hydroxyphenylpyruvic acid oxidation

oxidation of *p*-hydroxyphenylpyruvic acid to homogentisic acid is even today the least understood step of any in the scheme shown in Fig 12 6. So far it has not been possible to identify a free intermediate in this step. It appears as though the hydroxylation of the ring and the migration and oxidative decarboxylation of the side chain all take place as a complicated single step. This reaction is discussed in more detail in the chapter on tyrosinosis (Chap 11).

#### HOMOGENTISIC ACID A NORMAL INTERMEDIATE

The presence of homogentisic acid as a normal intermediate in the scheme deserves further comment. It should be recalled that in 1911 Dakin [79] suggested that not only was homogentisic acid an abnormal

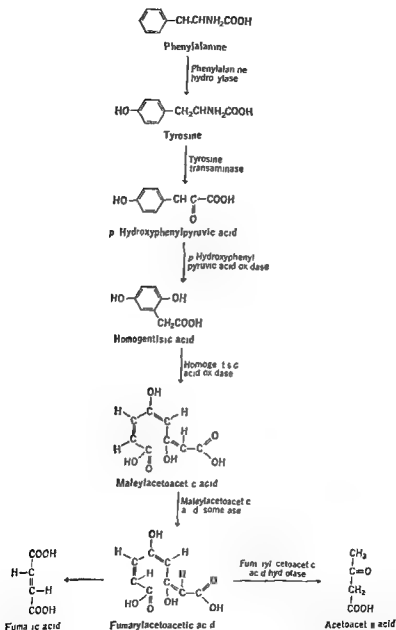


Fig. 17-6 Enzymatic steps in the oxidation of phenylalanine and tyrosine to acetoacetic acid

## METABOLIC DEFECT IN ALCAPTONURIA

Even though Garrod suggested in 1908 (11) that the metabolic defect in alcaptonuria was the absence of the liver enzyme catalyzing the oxidation of homogentisic acid other possible explanations have been offered from time to time by other workers. At the time Garrod presented his theory it was quite generally believed that alcaptonuria was due to the formation of homogentisic acid by intestinal organisms. This opinion was based upon an assumption of Wolkow and Baumann (10) that the synthesis of homogentisic acid from tyrosine or phenylalanine was too complicated to be accomplished by human tissues and therefore must have resulted from the action of bacteria in the gut.

In 1914 Gross (87) presented evidence for an enzymatic defect in alcaptonuria. He reported that homogentisic acid did not disappear when it was incubated with the serum from an alcaptonuric patient whereas it did disappear when incubated with the serum from normal individuals, presumably because of the presence in normal serum of a homogentisic acid-oxidizing enzyme. A few years later Katsch and Stern (88) suggested that it was not an enzyme that was missing in alcaptonuric serum but that the results of Gross were due to the presence of an inhibitor in alcaptonuric blood. Both these findings were disputed by Lanyar and Lieb (89) who pointed out that the previous workers had not controlled the experimental conditions satisfactorily, particularly the pH, and that autooxidation of homogentisic acid probably accounted for their results. Unfortunately the report that Gross found an enzyme missing in alcaptonuric blood is still widely quoted in medical texts. It is now certain that his results were in error since plasma contains no detectable homogentisic acid oxidase (33).

Another proposal as to the nature of the defect was that of Dakin (79) as discussed above in which he considered that the whole pathway to homogentisic acid in alcaptonuric patients is abnormal. More recently Neuberger (27) suggested that perhaps the alcaptonuric kidney differs from the normal kidney in having the capacity to secrete homogentisic acid actively while the normal kidney might not and that this unusual renal defect might account for the abnormal urinary product—see Dent (155). However throughout the last 50 years Garrod's suggestion that a liver enzyme is missing has been generally considered the most reasonable hypothesis. It was obvious that the net effect would be the same whether the enzyme itself or a vital cofactor is missing or whether there is an inhibitor of the enzyme in alcaptonuric liver.

A recent analysis of the enzymes involved in tyrosine metabolism in normal and alcaptonuric liver showed that only homogentisic acid oxidase is missing in alcaptonuric liver and that all the other enzymes involved in tyrosine metabolism to acetoacetic acid are present and have about



urinary product but that its formation resulted from the metabolism of tyrosine by an abnormal pathway in alcaptonuria. The main support for this proposal was that when he fed animals and an alcaptonuric patient [80, 81] derivatives of phenylalanine and tyrosine (*p*-methylphenylalanine and *p*-methoxyphenylalanine) which because of their para substituent, could not form quinol intermediates he found them to be well metabolized (in his view, by the normal pathway of tyrosine metabolism). He also found that these compounds caused an increase in acetoacetic acid when perfused through dog liver, as did phenylalanine and tyrosine. Although Dakin's results remained unexplained, through the years more and more evidence has accumulated that homogentisic acid is indeed a normal intermediate. For example it was shown that some homogentisic acid accumulated from tyrosine in rat liver homogenates under certain experimental conditions [82-70] more recently, it was shown that homogentisic acid accumulated quantitatively from tyrosine or *p*-hydroxyphenyl pyruvic acid in dog liver [82] and human liver [16] preparations in the presence of  $\alpha\alpha'$ -dipyridyl an inhibitor of homogentisic acid oxidase. Homogentisic acid oxidase is widely distributed in nature and where it occurs it is associated with the other enzymes involved in the oxidation of tyrosine to acetoacetic acid.

There still remained the problem of explaining Dakin's results. In 1957 Iarrung, Gottesman and Crandall [83] reinvestigated the ketogenic effect of the tyrosine analogues. They found that *p*-methoxy DL-phenylalanine is not ketogenic and that neither the L-form nor *p*-methoxyphenylalanine was metabolized although the D isomer was deaminated in dog liver. They attributed the earlier results of Wakeman and Dakin [81] to the ketogenic effect of the ammonium ion released by the deamination of the D-component. (The ketogenic effect of ammonium ion [84] was not known in 1911.) It must also be inferred from these results that Dakin did not have sufficiently sensitive methods to detect the excretion of the unchanged compounds in the urine. The present scheme of tyrosine oxidation (Fig. 12.6) is therefore very much like that of Neubauer (Fig. 12.2) in the steps leading to the formation of homogentisic acid with the notable exception that neither 2,5-dihydroxyphenylalanine nor 2,5-dihydroxyphenylpyruvic acid is now considered a likely intermediate. Both these compounds produced extra homogentisic acid when fed to alcaptonuric patients [18-27] but they were found to be inactive as substrates when tested with mammalian liver preparations which oxidize tyrosine or *p*-hydroxyphenylpyruvic acid to homogentisic acid [16-32, 82-85]. These results also exclude the alternative pathway suggested by Neuberger in 1947 in which tyrosine would be oxidized first to 5-dihydroxyphenylalanine and then either through 5-dihydroxyphenylpyruvic acid or 5-dihydroxyphenylethylamine to homogentisic acid [86].

evident 38 hr after birth although after 52 hr they were deeply stained and continued to be thereafter. The reason for the delay in the excretion of homogentisic acid in newborn alcaptonuric patients is probably that the enzyme systems involved in tyrosine oxidation are not completely developed at birth and that they increase in activity during the next few days [94]. Once established the defect continues relentlessly throughout life. No therapeutic agent has been found which substantially alters the degree of the defect. The amount of homogentisic acid excreted per day is usually from 4 to 8 gm; it can be altered by changing the content of phenylalanine and tyrosine in the diet. In starvation there is a marked decrease in homogentisic acid excretion [95] as would be expected although Mittlebach [96] showed that on a diet very low in protein the alcaptonuric patient continued to excrete some homogentisic acid presumably from the breakdown of tissue proteins.

#### HOMOGENTISIC ACID OXIDASE

The enzymatic step which is missing in alcaptonuria is the further metabolism of homogentisic acid by an oxidative cleavage of the ring to yield maleylacetoacetic acid [97] which in turn is isomerized enzymatically to fumarylacetoacetic acid [98-100] (Fig. 12.6). The next step is hydrolysis to fumaric and acetoacetic acids by an enzyme which appears to be the same as that shown to hydrolyze a number of  $\alpha$ - $\gamma$ -diketo acids by Meister and Greenstein [100a] and to hydrolyze triacetic acid [101-102].

In 1961 Suda and Takeda [103] solubilized an enzyme from a strain of *Pseudomonas* adapted to tyrosine which catalyzed the oxidation of homogentisic acid; they named it *homogentisicase*. They then studied the properties of a similar enzyme from rabbit liver [104]. Homogentisicase or as it is more generally called homogentisic acid oxidase has been purified to some degree in several laboratories and many of its properties have been described [97, 105-107]. It belongs to the class of oxygenases. In the cleavage of the benzene ring both oxygen atoms come from atmospheric oxygen as indicated recently in experiments with  $O^{18}$  [108]. The enzyme contains essential sulfhydryl groups and requires ferrous iron [104, 109] as do several of the other oxygenases involved in ring cleavage reactions such as pyrocatechase [110-111], hydroxyanthranilate oxidase [112] and protocatechuic acid oxidase [113-114] (see Macon [115] p. 126). No other cofactors have been clearly implicated in this reaction. Although evidence that one did exist was presented by Suda and Takeda [104] in 1960 it now appears that a protective effect of glutathione may account for the earlier results [116]. There is also general agreement that the previously suggested requirement for ascorbic acid in this enzyme system [117] is an indirect one due to the requirement for ferrous iron. The only function that has been demonstrated for ascorbic acid in this reaction is to maintain iron in the reduced form [104, 118].

the same activity as in normal liver [16, 38] (Table 12 1) Evidence was also obtained that the lack of activity is not due to the presence of inhibitor or to the lack of any known cofactor [16] It now seems reasonable to define the defect in alcaptonuria as the failure to synthesize active homogentisic acid oxidase and to attribute all the findings in alcaptonuria to this specific enzymatic defect Whether these individuals form a catalytically inactive protein differing perhaps only slightly in structure from active homogentisic acid oxidase, or whether they produce no protein at all resembling the enzyme is still unknown

TABLE 12 1 ACTIVITY OF TYROSINE OXIDATION ENZYMES IN ALCAPTONURIC AND NONALCAPTONURIC HUMAN LIVER HOMOGENATE

| Enzymes                             | Enzyme activity $\mu$ moles of substrate oxidized/hr/gm of liver |              |
|-------------------------------------|--|--------------|
|                                     | Nonalcaptonuric  | Alcaptonuric |
| Tyrosine transaminase               | 16   | 32           |
| p-Hydroxyphenylpyruvic acid oxidase | 67   | 46           |
| Homogentisic acid oxidase           | 268  | <0.048       |
| Maleylacetoacetic acid isomerase    | 960  | 780          |
| Fumarylacetoacetic acid hydrolase   | 298  | 22           |

Units calculated as  $\Delta$  log optical density per hour per 0.1 gm wet weight of liver [38]

SOURCE: B. V. La Du et al [16]

A recent opportunity to obtain at autopsy samples of kidney tissue from an alcaptonuric patient made it possible to show that homogentisic acid oxidase is also absent in alcaptonuric kidney but that the other enzymes involved in the oxidation of tyrosine to acetoacetic acid can be easily detected [90] Homogentisic acid oxidase could be demonstrated in nonalcaptonuric human kidney autopsy samples Thus the genetic defect in alcaptonuria is not limited to the synthesis of homogentisic acid oxidase in liver, it appears to affect the synthesis of the enzyme wherever it is normally present This is of theoretic interest, from the viewpoint of the genetic control and tissue specificity of enzymes [91-92] and it may be of some practical value in the detection of the carrier trait in relatives of alcaptonuric patients It might be possible to show a decreased amount of enzyme in carriers of the trait by a direct assay of the enzyme in a tissue more accessible than liver or kidney This would perhaps be a more accurate means to measure the enzyme than to determine it indirectly by the rate of metabolism of homogentisic acid in a tolerance test

The metabolic abnormality in alcaptonuria is present essentially from birth Garrod noted in 1901 [93] that staining of the diapers was scarcely

## INTERMITTENT ALCAPTONURIA

There are a few reports in the literature of intermittent alcaptonuria or instances in which it is reported that alcaptonuria has spontaneously disappeared—see Galdston et al [40]. In view of the finding that alcaptonuria is associated with the lack of a specific enzyme it is difficult to imagine how this hereditary condition would undergo intermittent exacerbations and remissions or a spontaneous cure. Perhaps some of these cases of alcaptonuria are misdiagnosed and have some other reducing substance present in the urine. Other cases may be examples in which some agent such as those described below which can induce experimental alcaptonuria in animals has altered the activity of the enzymes in this pathway with resultant homogentisic acid excretion. Any further cases of alcaptonuria of this type should be carefully investigated with the specific methods now available to establish beyond any doubt that homogentisic acid is the reducing substance excreted in the urine. In 1948 Fishberg reported [130] that a patient with autotoxic enterogenous cyanosis excreted up to  $\frac{1}{2}$  gm per day of benzoquinone acetic acid the quinone corresponding to homogentisic acid. The amount excreted varied in inverse ratio to the ascorbic acid excretion. She also found on the basis of nonspecific tests that patients with rheumatic fever and with scurvy excrete a similar quinone capable of producing methemoglobinemia. These results have been questioned by Consden et al [131] who found no benzoquinone acetic acid excreted in scorbutic guinea pigs or in patients with rheumatic fever. They suggest that bacterial activity in the urine leading to nitrite formation may have been responsible for the results of the qualitative tests employed by Fishberg. The excretion of benzoquinone acetic acid in the case of enterogenous cyanosis cannot be explained in this way. They suggest that the product might have been homogentisic acid which was oxidized to the quinone by nitrite arising from bacterial activity. Others have found nitrite in the urine and blood of patients with enterogenous cyanosis [132].

## METABOLISM OF HOMOGENITISIC ACID

Under normal conditions no homogentisic acid is present in the urine and none can be detected in plasma by the methods now available [70]. Leaf and Neuberger [133] found that feeding as much as 5 gm homogentisic acid to normal adults produced no homogentisic aciduria. However they found a transitory alcaptonuria following the intravenous injection of 13 or 10 gm homogentisic acid. In these experiments the plasma concentration never rose above 1.5 mg per 100 ml plasma and it returned to normal values within 30 min.

One might expect to find elevated plasma levels of homogentisic acid in alcaptonuric patients in view of the large quantity of this acid ex-

Homogentisic acid oxidase is inhibited by various quinones [106] by sulfhydryl binding agents [109] and by metal chelating agents such as  $\alpha, \alpha'$ -dipyridyl and *o* phenanthroline [119], which react with ferrous iron

Homogentisic acid oxidase activity can be measured manometrically, since the oxidation requires the uptake of two atoms of oxygen [97, 120] or it can be measured spectrophotometrically [97] by following the absorption of the product maleylacetoacetic acid at 330 m $\mu$ , providing the product is stable under the assay conditions. The enzyme is found in the soluble fraction of liver and kidney [121] as are all the mammalian enzymes involved in the conversion of tyrosine to acetoacetic acid [122]. Homogentisic acid oxidase activity is highest in liver, there is less activity in kidney, and no significant activity has been found in any of the other tissues so far examined [107-123] such as blood, salivary glands, germinal epithelium and muscle. This general distribution pattern has been found in rat, rabbit, guinea pig, and pigeon [107]. In man it is also highest in liver [16] and appreciable activity is also present in kidney [90]. The liver of the toad, *Bufo marinus* has as high homogentisic acid oxidase activity as that found in mammalian liver [124]. The presence of the enzyme in some microorganisms adapted to tyrosine has been previously mentioned [103, 125].

The optimal pH for this enzyme is about 7.0 [104, 105] and it is specific for homogentisic acid. Closely related compounds such as *o*-hydroxy phenylacetic acid, *p*-hydroxyphenylacetic acid and gentisic acid are not oxidized [105] nor are homogentisic acid ethyl ester and homogentisic acid lactone. The quinone formed by oxidizing homogentisic acid [127] does not appear to be an intermediate in the oxidation [105] and in fact this quinone is an inhibitor of the enzyme [105, 126]. The requirement for ferrous iron is apparently specific, since other bivalent metals such as  $\text{Co}^{++}$ ,  $\text{Zn}^{++}$ ,  $\text{Mg}^{++}$  and  $\text{Mn}^{++}$  cannot replace it [104, 126].

A report in the German literature [128] that gentisic acid was less well metabolized in an alcaptonuric patient than in the normal person led Garrod to conclude that the enzyme system defective in alcaptonuria must catalyze the oxidation of some other 2,5-dihydroxyphenyl compounds as well as homogentisic acid [1]. The specificity of the enzyme rules out this possibility and the reason for the results in the original study is not known. It is possible that a larger percentage of gentisic acid is excreted as the free acid and less as conjugated derivatives in the alcaptonuric patient because of some inhibitory effect of homogentisic acid on the conjugation of gentisic acid and that for this reason less gentisic acid appeared to be metabolized. However in one attempt to confirm this finding the excretion of free gentisic acid was found to be about the same in an alcaptonuric patient as in nonalcaptonuric persons [129].

of generalized ochronosis in the bones and connective tissues of cattle dogs and horses in which the tissues are described as being black as coal but again homogentisic acid has never been identified in the urine with certainty [20 136 137]

Experimental alcaptonuria has been produced in rats and mice by feeding large quantities of phenylalanine or tyrosine (see Table 12.2). It is also reported that vitamin C deficient guinea pigs fed extra phenylalanine [118 138] or tyrosine [118] excreted homogentisic acid as well as *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid. Other workers however have found only the latter two compounds and no homogentisic acid in similar experiments with vitamin C-deficient guinea pigs [139]. It should be noted that in some experiments of this type the claim has been made that homogentisic acid was excreted even though the analytical methods employed would not distinguish between *p*-hydroxyphenylpyruvic acid and homogentisic acid [140].

Experimental alcaptonuria has been produced in rats and mice by feeding large quantities of phenylalanine and tyrosine (see Table 12.2).

TABLE 12.2 METHODS OF PRODUCING EXPERIMENTAL ALCAPTONURIA

| Agent                                | Species     | Comment  | Reference |
|--------------------------------------|-------------|--|-----------|
| Feeding L-phenylalanine              | Rats        |  | [168 170] |
|                                      | Mice        |  | [111]     |
|                                      | Guinea Pigs | Excreted HGA and 1 HPP on vitamin C-deficient diet defect corrected by vitamin C [118]   | [138 118] |
| Feeding L-tyrosine                   | Rats        | Believed by authors to be due to adaptive increase in tyrosine transaminase activity but decrease in homogentisic acid oxidase activity also found | [145]     |
|                                      | Guinea pigs | On vitamin C-deficient diet other workers find only PHPI 1 HPI no HGA [139]<br>Defect corrected by ascorbic acid [118] or folic acid [111 13]      | [117 118] |
|                                      | Human being | Large doses over 1 day 50 gm 150 gm until on HGA excreted  | [141]     |
| Diet deficient in sulfur amino acids | Rats        | Effect reversed by cysteine—not by ascorbic acid   | [14 145]  |
| αα-Dipyridyl                         | Guinea pigs | Defect not altered by ascorbic acid  | [104 174] |

NOTE HGA = homogentisic acid PHPI = *p*-hydroxyphenylpyruvic acid 1 HPI = *p*-hydroxyphenyllactic acid

creted per day. Neuberger, Rimington and Wilson [27] found however that in a 7 year-old alcaptonuric girl the fasting plasma level was not more than about 8 mg per 100 ml plasma and that this level did not increase significantly following the oral administration of 3 gm L-phenyl alanine. Nevertheless within 6 hr approximately 85 per cent of the given amino acid could be accounted for as homogentisic acid in the urine. Neuberger et al. made a very significant observation regarding the excretion of homogentisic acid during this investigation. The plasma clearance data indicated that unless a large fraction of the urinary homogentisic acid were both synthesized and excreted within the kidney glomerular filtration alone could not begin to account for the rate of homogentisic acid excretion. In fact the clearance approached 400 to 500 ml per min. about equal to the renal blood flow. Even though it is most unusual for a normally occurring intermediate to be actively secreted by the kidney, this seems to be true of homogentisic acid. This conclusion is in agreement with the earlier observation of Katch and Metz [184] that intravenous homogentisic acid given to an alcaptonuric subject did not increase the plasma concentration significantly.

More recent experiments in the author's laboratory [33] using an enzymatic assay to estimate plasma homogentisic acid have confirmed these conclusions. Alcaptonuric and nonalcaptonuric individuals were found to excrete homogentisic acid rapidly after oral administration and the renal clearance data indicated active secretion by the kidney. The possibility [27, 135] that there might be an important difference in the renal handling of homogentisic acid between normal individuals and alcaptonuric patients can be dismissed.

It appears that two factors serve to keep the plasma and presumably the tissue concentrations of homogentisic acid at a low level: the great capacity to metabolize this acid in the liver and kidney and the rapid renal tubular secretion of homogentisic acid. Even in the alcaptonuric patient the renal mechanism is capable of effectively lowering the plasma level when homogentisic acid is given. This defense mechanism may be highly significant in view of the many years required for ochronosis to appear. It is quite possible that in the alcaptonuric person the tissues are only occasionally flooded with homogentisic acid and that this event has to be repeated many times over a period of years before tissue pigmentation occurs to a significant extent.

#### EXPERIMENTAL ALCAPTONURIA

Spontaneous alcaptonuria has not been found in any species except man. Although there is a report by Lewis [136] of a rabbit with urine that darkened upon exposure to air and gave some of the qualitative tests for homogentisic acid, the latter was never isolated or positively identified and the rabbit died without offspring. There are also reports

by injecting rats intraperitoneally with L-tyrosine. Under these conditions the activity of the enzymes later in the pathway was not changed significantly. This adaptation is unusual, since it can also be produced by injecting hydrocortisone, by certain other amino acids and by propylene glycol [123]. They also found that injections of L-tyrosine were not effective in adrenalectomized animals unless hydrocortisone was also given.

In none of the methods for producing experimental alcaptonuria is the block in homogentisic acid oxidation complete. In most only a small percentage of the homogentisic acid formed per day is excreted. None of the methods is well suited for long term studies to produce experimental ochronosis and possibly arthritis secondary to alcaptonuria.

### HEREDITARY ASPECTS

The first paper describing the inheritance of alcaptonuria was that of Garrod in 1902 [14] in which he presented evidence that this condition is congenital and familial and that it occurs more often in families in which there are consanguineous marriages. He suggested that alcaptonuria might be transmitted as a single recessive Mendelian trait. He believed that homogentisic acid arose in the normal course of tyrosine metabolism and that consanguineous marriages brought to light a recessive defect in this metabolic process. In 1902 Bateson and Saunders [147] also suggested that the inheritance of a rare recessive factor might explain the incidence of alcaptonuria. These studies on the mode of transmission of alcaptonuria were among the first on hereditary metabolic diseases.

Although in the years immediately following examples were recorded in which direct transmission (i.e. parent and offspring affected) of alcaptonuria occurred, Garrod believed that these were examples of a heterozygous individual mating with a homozygote. As more family histories were described the general opinion of the recessive nature of the disease remained unchallenged. In 1932 Hogben, Worrall and Zieve [148] carefully summarized all the known cases of alcaptonuria reported up to that time. Again the recessive character of the disease was confirmed in nearly all the families, and it was observed that at least half the affected individuals were the offspring of consanguineous matings. Although there was an unequal sex distribution in their cases—100 males and 46 females—they did not consider this an indication that the condition was semilethal in females. They noted that males were more often the probands in affected families and suggested that the higher incidence in males might be because of the more frequent examination of males. They also noted that among infants there were slightly more females than males, which was in agreement with this explanation. Nevertheless it is frequently stated in medical texts that the incidence of alcaptonuria is twice as high



There is also a report of transitory homogentisic acid excretion after feeding a human volunteer large amounts of L-tyrosine [141]

Another type of experimental alcaptonuria has been induced in rats by a diet deficient in the sulfur-containing amino acids [142] This was not corrected by giving ascorbic acid but was reversed by giving cysteine [143] In this type of experimental alcaptonuria, proportionately less *p* hydroxyphenylpyruvic acid is excreted than in the type that responds to ascorbic acid [118]

In addition to the above methods the finding that homogentisic acid oxidase requires ferrous iron and can be inhibited by  $\alpha$   $\alpha'$ -dipyridyl was used by Suda and Takeda [104] to induce experimental alcaptonuria in guinea pigs They injected  $\alpha$   $\alpha'$ -dipyridyl and fed extra tyrosine The excretion of homogentisic acid in these animals was not corrected by administration of vitamin C

In another investigation, it was found that human volunteers on a vitamin C-deficient diet for several months with frank scorbutic symptoms did not excrete increased amounts of urinary phenols when given 20 gm tyrosine orally [144] This is further evidence that there is no direct requirement for ascorbic acid in homogentisic acid oxidation It can be concluded that the majority of instances of experimental alcaptonuria are either the result of direct inhibition of homogentisic acid oxidase or are due to an imbalance in the various enzyme reactions sufficient to cause an accumulation of homogentisic acid and its urinary excretion

Lin and Knox [145] have recently reported that in experimental alcaptonuria in rats induced by a diet supplemented with extra tyrosine no homogentisic acid was excreted for the first 3 or 4 days Following this initial lag period the degree of homogentisic acid excretion increased during the next several weeks They attribute these findings of the gradual increase in intensity of the alcaptonuria to an adaptive increase in tyrosine transaminase activity They believe that this type of alcaptonuria occurs because the relative rate of homogentisic acid formation overbalances the rate of homogentisic acid degradation Their data also show a significant decrease in liver homogentisic acid oxidase in the experimental group the decrease may be an important contribution to the resulting alcaptonuria

It is of interest to recall that the activity of the enzymes in the pathway following homogentisic acid oxidase was approximately normal in alcaptonuric liver (Table 12-1) Since the only known endogenous source of maleylacetoacetic acid is the oxidation of homogentisic acid, it appears that normal levels of the isomerase are produced in the absence of its substrate Thus it is a constitutive rather than an adaptive enzyme

Further studies should be mentioned in connection with the 'adaptive changes in the activity of liver tyrosine transaminase Lin and Knox [146] found that the level of this transaminase could be increased several fold

It would be most helpful if a diagnostic test were available to detect the carrier state. In view of the nature of the enzymatic defect in alcaptonuric individuals one might hope to find approximately one half of the normal amount of enzyme in the tissues of heterozygous individuals as is apparently the situation in phenylketonuria [153-155] and galactosemia [156-157]. Measurements of the ability to metabolize homogentisic acid

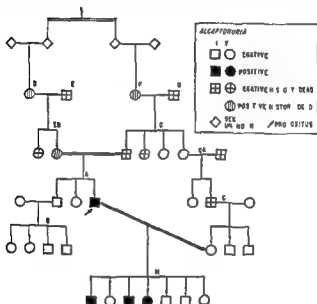


Fig 199 Alcaptonuria in a Lebanese family reported by Ismail Adnan and Abu Feisal (By permission of Ismail Adnan et al [150])

by an oral homogentisic acid tolerance test have so far shown no difference between relatives of alcaptonuric patients and normal controls [158]. These results may be due to the tremendous capacity of the liver (see Table 12.1) to metabolize this acid. Perhaps even if this reserve were reduced to one half it might not be detected by an oral tolerance test. In fact, assuming a liver weight of 1,500 gm and assuming that the liver homogentisic acid oxidase is as efficient *in vivo* as under assay conditions *in vitro*, it can be calculated that the normal adult liver can metabolize over 1,600 gm homogentisic acid per day.

## TREATMENT

Attempts to treat alcaptonuria have been directed either toward correcting the underlying metabolic defect or preventing or reversing the

in males as in females. The paper by Hogben et al. is obviously the source of this information.

Among the families reviewed by Hogben et al. there were some in which a dominant form of alcaptonuria had to be considered. In a family studied by Pieter [149] the author felt compelled to conclude that a dominant type of alcaptonuria existed. In the end this conclusion depended upon the predicted opportunity for marriage between homozygous and heterozygous individuals and the frequency of the heterozygote in the general population or perhaps more exactly the incidence of the heterozygote within a selected population. The incidence of alcaptonuria in the general population can be only roughly estimated. At least 200 cases have been described but this is a conservative number since new cases are generally not reported unless there are some other special features present. It is reasonably certain however that alcaptonuria is less rare than was believed 30 years ago. It is reported [9] that in a recent study in northern Ireland by A. C. Stephenson the incidence of alcaptonuria is from 3 to 5 per million individuals. This would give a considerably higher incidence of heterozygous individuals in the general population than assumed by Hogben et al. [148].

It is important to recall that in the families in which direct transmission of alcaptonuria has been found the number of consanguineous marriages is very high. One of the best examples of this is the recently described kindred by Khachadurian and Abu Faisal [100]. In this Lebanese family there was a total of seven alcaptonuric patients in four successive generations (Fig. 12.9). Careful investigation however showed that the grandmothers of the proband were first cousins and that at least two consanguineous marriages existed in this family. This pedigree is particularly instructive because it illustrates how a recessive trait could appear to be a dominant one unless the entire family pedigree is known.

At present most if not all cases appear to represent the inheritance of a single autosomal recessive gene. This is supported by the biochemical finding that a single enzyme system is inactive in this condition and that only one clinical form of alcaptonuria is known. The few cases in which it has been considered as possibly a dominant form have not shown any clinical differences from the majority of cases.

The suggestion by Milch [151-152] that alcaptonuria is inherited as a dominant gene with incomplete penetrance seems unnecessarily complicated to explain the data at hand—see Knox [9]. In fact while the possibility of a dominant type of alcaptonuria cannot be excluded it should be pointed out that no convincing evidence for it has yet been presented.

The fact that a rare disease with recessive inheritance may be encountered more frequently in selected inbred populations complicates the estimation of the incidence of heterozygotes in the general population.

It would be most helpful if a diagnostic test were available to detect the carrier state. In view of the nature of the enzymatic defect in alcaptonuric individuals one might hope to find approximately one half of the normal amount of enzyme in the tissues of heterozygous individuals as is apparently the situation in phenylketonuria [153-155] and galactosemia [156-157]. Measurements of the ability to metabolize homogentisic acid

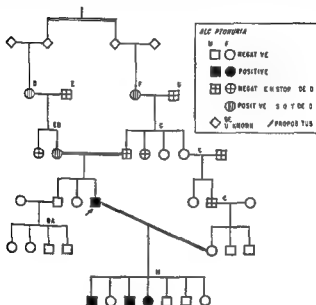


Fig 129 Alcaptonuria in a Lebanese family report 1 by Khachadurian and Abu Feisal (By permission of A Khachadurian et al [150])

by an oral homogentisic acid tolerance test have so far shown no difference between relatives of alcaptonuric patients and normal controls [158]. The results may be due to the tremendous capacity of the liver (see Table 12.1) to metabolize this acid. Perhaps even if this reserve were reduced to one-half it might not be detected by an oral tolerance test. In fact, assuming a liver weight of 1,500 gm and assuming that the liver homogentisic acid oxidase is as efficient *in vivo* as under assay conditions *in vitro*, it can be calculated that the normal adult liver can metabolize over 1,600 gm homogentisic acid per day.

## TREATMENT

Attempts to treat alcaptonuria have been directed either toward correcting the underlying metabolic defect or preventing, or reversing the

pigmentation and arthritic changes Galdston Steele and Dobriner [40] administered several vitamins brewers yeast tyrosinase insulin and adrenocortical extract without altering the amount of homogentisic acid excreted by an alcaptonuric patient Several groups have studied the effectiveness of vitamin C [26, 27, 40, 159, 160] Although it corrects the alcaptonuria induced in guinea pigs by feeding large amounts of tyrosine [117] it does not change the hereditary type of alcaptonuria

Other agents, such as vitamin B<sub>12</sub> [161], cortisone [19 162, 163] and phenylbutazone [164], are without influence on the metabolic defect A confusing report by Cope and Kassander [56] that cortisone corrected the metabolic error is difficult to interpret since the authors claim this was a case of ochronosis without homogentisic acid in the urine

Now that it is certain that the basic defect is the lack of a specific enzyme replacement of the missing enzyme is theoretically a therapeutic measure to consider, but this is not practical at present Even if pure homogentisic acid oxidase were available in large quantities its administration might very well cause an antibody response capable of inactivating the enzyme It is possible that a synthetic chemical compound might be found which could replace the missing enzyme, but no such catalyst is now available

In the past it has been suggested that dietary phenylalanine and tyrosine be reduced to decrease the output of homogentisic acid A severe restriction of the intake of these amino acids is not practical except for brief periods and might be dangerous to the patient if continued over a long time

Since from a practical standpoint the importance of the metabolic defect is mainly that it leads to pigmentation and arthritic changes therapeutic measures could be aimed primarily at preventing or correcting these complications of the disease It is possible as Sealock Galdston and Steele have pointed out that large amounts of ascorbic acid might prevent the deposition of ochronotic pigment [26] even though this does not alter the metabolic defect

## SUMMARY

1 Alcaptonuria is a rare hereditary metabolic disease in which homogentisic acid an intermediary product in the metabolism of phenylalanine and tyrosine cannot be further metabolized The metabolic defect causes a characteristic triad of homogentisic aciduria, ochronosis and arthritis

2 The cause of the disease is a constitutional lack of the enzyme homogentisic acid oxidase This enzyme normally exists primarily in the liver and kidney It requires oxygen ferrous ion and sulphydryl groups for opening the ring of homogentisic acid

3 The condition is inherited as an autosomal recessive disease. No method for the detection of heterozygotes has been devised.

4 The relationships between the metabolic defect and the complications ochronosis and arthritis remain a challenging research problem of the future. Even though the lack of homogentisic acid oxidase is no doubt the ultimate cause of the complications, the mechanisms which bring them about are unknown.

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## Chapter 13

### Albinism\*

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Thomas B Fitzpatrick

Albinism is an inherited disorder of melanin metabolism characterized by a decrease or absence of melanin in the skin, hair and eyes. Because the clinical features are so striking the condition was recognized early, and accurate descriptions of albinos are found in the writings of Pliny and Ptolemy. The term *albino* is derived from the Latin adjective *albus* white, and was first used by Balthazar Telles for certain white Negroes seen by him and other traders on the African coast. An historical review of albinism can be found in the monographs by Pearson, Nettleship and Usher [1] (1911-1913) and a more recent well-documented summary by Irogatt [2]. The essential clinical features of albinism are well described in the following paragraphs written by Lionel Wafer [3] in 1699.

There is one complexion so singular that I never saw nor heard of any like them in any part of the world they are white tis a milk white lighter than the colour of any Europeans and much like that of a white horse their seeing so clear as they do in a moon shiny night we used to call them moon-eyed. For they see not very well in the sun poring in the clearest day (their eyes being but weak and running with water if the sun shine towards them) so that in the day time they care not to go abroad when the moon shiny nights come they are all life and activity running abroad and into the woods (skipping about like wild bucks and running as fast by moon light) even in the gloom and shade of the woods as the other Indians by day being as nimble as they tho not so strong and lusty (neither is the child of a man and woman of these white Indians white like the parents but copper coloured as their parents were) and they were but short in d

Outstanding characteristics are the milk white color and marked photophobia with eyes being but weak and running with water if the sun shine towards them. The recessive inheritance is suggested by

This chapter was written while the author was in Oxford, England as a Fellow of the Commonwealth Fund

neither is the child of a man and woman of these white Indians white like the parents but copper coloured. The marked susceptibility of albinos to skin cancer with metastases and death is expressed in the phrase 'they were but short lived'. The striking contrast to the normal dark skin in Indians and Negroes set them apart and many myths were conjured up about these strange 'moon-eyed' people, "skipping about like wild bucks, and running as fast by moon light". Even today

in Panama in the mythology of primitive Indian tribes there are several prominent albino heroes and albinos are believed to have a special aura.

Garrod [4] with judicious prescience included albinism among his inborn errors of metabolism. His speculations on the nature of the metabolic defect in albinism and the mechanism of melanin formation are truly remarkable.

Three possible explanations of the phenomenon of albinism suggest themselves. We might suppose that the cells which usually contain pigment fail to take up melanins formed elsewhere or that the albino has an unusual power of destroying these pigments or again that he fails to form them.

It is very unlikely that the melanin is conveyed to the pigmented cells and there deposited for all the evidence indicates that the pigment is formed *in situ* probably by the action of intracellular enzymes.

Only certain specialized cells appear to have the power of forming melanin.

Taking all the known facts into consideration the theory that what the albino lacks is the power of forming melanin which is normally possessed by certain specialized cells is that which has most in its favour and is probably the true one. If so an intracellular enzyme is probably wanting in subjects of this anomaly an explanation which brings albinism into line with some other inborn metabolic errors of which a similar explanation is at least a possible one [4].

This chapter will be largely devoted to a proof of Garrod's theory based on present knowledge of melanin formation. For purposes of orientation before discussing the biochemical lesion of albinism the characteristic clinical features of the various types of albinism will be described.

## DEFINITION AND CLASSIFICATION

Albinism is an inherited metabolic defect of a pigment cell or *melanocyte* resulting in a failure of this cell to form melanin.

The melanocyte is a specialized distinctive dendritic shaped cell with two or more cytoplasmic processes surrounding a perikaryon. Melanocytes are derived from the neural crest<sup>1</sup> and are normally located

The retinal pigment epithelial cells are derived from the outer layer of the optic cup. The pigment cells have a morphology different from that of melanocytes elsewhere. Electron micrographs of the melanin granules of the retinal pigment epithelium show a morphology similar to that of melanin granules of the hair bulb. The retinal pigment epithelium contains tyrosinase but only during the first few weeks of development [5].

in man in certain characteristic regions *skin* (at the epidermal-dermal junction) *hair bulb nervous system* (in the paraneuron) and *eye* (uveal tract and retinal pigment epithelium). The melanocytes constitute an embryologic, morphologic and biochemical unit referred to as the *melanocyte system* (12, 13, 14). With the curious exception of the melanocytes of the hair bulb and retinal pigment epithelium, all melanocytes have the propensity to form malignant melanomas.

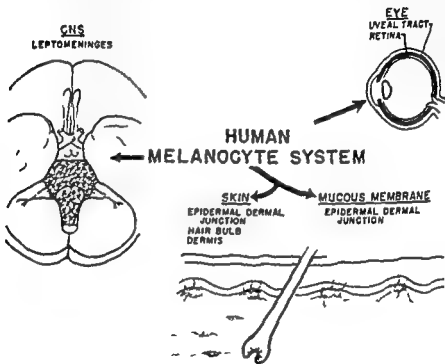


Fig. 13.1 The human melanocyte system

The metabolic defect of the melanocyte in albinism may involve the entire melanocyte system or only one locus of melanocytes. For example, albinism may be limited to the retinal pigment epithelium. Classification therefore should refer to the *extent* of the involvement of the melanocyte system. Numerous terms have been used including complete and incomplete, perfect and imperfect and partial. Table 13.1 gives the terminology recommended here and some of the terms which have been used. Since the adjectives describe the extent of the metabolic defect throughout the melanocyte system, the terms *universal*, *generalized* and *localized* are more acceptable than complete, imperfect, partial, etc. [1, 6, 7].

In order to classify albinism as *universal*, histologic examination of the entire melanocyte system must be made to prove the absence of melanin.

TABLE 13.1 CLASSIFICATION OF ALBINISM

| <i>Recommended terminology</i>    | <i>Present terminology</i>                       | <i>Description</i>   | <i>Heredit</i>                            |
|-----------------------------------|--|--|---|
| Universal albinism                | Complete albinism<br>Perfect albinism            | Absence of melanin in the hair bulb skin uveal tract and retinal pigment epithelium  | Autosomal recessive                       |
| Generalized albinism              | Incomplete albinism<br>Imperfect albinism        | Absence of melanin in the skin hair and retinal pigment epithelium but presence of melanin in the iris and occasionally the skin | Autosomal recessive<br>Irregular dominant |
| Localized albinism<br>Of the skin | Partial albinism<br>Piebaldism<br>White forelock | Absence of melanin in scattered isolated areas of skin and hair  | Autosomal dominant                        |
| Of the eye                        | Ocular albinism                                  | Absence of melanin in the hair of the frontal region<br>Absence of melanin in the retinal pigment epithelium                     | Sex linked recessive                      |

Affecting the whole of something specified. *The Shorter Oxford English Dictionary* Oxford University Press 1956

A clinical diagnosis of universal albinism cannot be made. It occurs in animals but none of the autopsy studies on human albinos have proved universal absence of melanin in man.

A diagnosis of *generalized albinism* requires that there be ocular involvement (nyctalopia photophobia decreased visual acuity and translucent irises).

## CHARACTERISTICS IN MAN

### INCIDENCE

Albinism is widespread in the animal kingdom. It is found in fish, birds and mammals [8, 9, 10].

Generalized albinism in man is not uncommon. The incidence has been reported to be from 1/5000 to 1/25000. The highest incidence (1/1000) was found in the San Blas Indians of Panama [11]. There are approximately 700 pedigrees of generalized albinism in the literature. It has been reported in all races.

The incidence of localized albinism of the eye, skin or hair is not known. Approximately twenty-four families with localized cutaneous and pilary albinism (*partial albinism*) had been reported as of 1954 [12].

Both generalized and localized albinism are usually recognized by the dermatologist who is consulted because of solar intolerance or by the ophthalmologist who is consulted because of ocular problems. Since many



generalized albinos do not seek the attention of these specialists, the incidence may be considerably higher than existing figures indicate

### CLINICAL FEATURES

#### *Universal and generalized albinism*

The generalized albino exhibits milk white skin color and whitish yellow hair, and has a characteristic facies because of photophobia and habitual squinting (Fig 13 2)



Fig 13 2 Generalized albinism in a Negro

**Skin Color** In all races (Caucasian Asiatic, Indian and Negroid), the skin color of the albino is milk white. The reflectance of Caucasian albino skin as measured by a reflectance meter [13] is no greater than that of untanned normal skin. In the African Negro albino, Barnicot [14] compared the color visually to that of a lightly tanned European, while in the case of African albino infants the skin is "very white."

In the Negroid race particularly there is a peculiar inherited pigmentary change known as "xanthism." The hair has a strong red color and the skin is a bright copper red. The iris color is diluted. Barnicot [15] believes that the condition is distinct from generalized albinism but Pearson et al [1] cite a pedigree in which both albinism and xanthism appear.

**Response to Solar Exposure** In both the Caucasian and Negro albino the skin of the exposed areas may be markedly erythematous, and in the African albino it may be wrinkled and folded particularly on the back of the neck and arms [14]. This change in surface contour is absent in African albino infants and thus may result from chronic solar exposure. In adult albinos exposed to prolonged intense solar radiation precancerous keratoses and squamous and basal carcinomas are almost invariably found [16]. Carcinoma of the exposed surfaces is extremely rare in pigmented peoples (Asiatic Indian and Negro). The demographic data of MacDonald [17] and others [18] strongly implicate solar ultraviolet radiation as a carcinogenic agent in the Caucasian. The susceptibility of the human albino skin and albino mouse to solar and artificial ultraviolet radiant energy and the relative immunity of pigmented peoples and

pigmented mice [19] indicate the important role of melanin. Thomson [20] has demonstrated the remarkable opacity of isolated Negro corneum to solar radiation.

The skin of the albino does not tan after exposure to ultraviolet light. The erythema threshold of unexposed skin is lower than in normal white skin. By carefully graded exposures the albino can achieve a fair degree of tolerance to solar exposure by a thickening of the stratum corneum.



Fig 13-3 Dermal melanocytosis nevus in generalized albinism (same patient as in Fig 13-2)

**Physiologic Responses of Albino Skin** There have been few if any studies of various physiologic responses of albino skin. Information is needed on wound healing, percutaneous absorption, sensory function, sweat and sebaceous gland secretion, insensible water loss, pH, circulation, and vascular reactions, thermoregulatory mechanisms, and electrical behavior. At present the sole recognized abnormal property of albino skin is the absence of melanin pigment. Albinos do not become pigmented during pregnancy, and the nipples remain pink [21].

**Pigmented Lesions in Albino Skin** Albino skin contains melanocytes which can proliferate into benign nonpigmented and pigmented nevi [22] (Fig 13-3). There are three case reports of malignant melanomas arising in melanocytes of albino skin [23-25].

In addition to pigmented and nonpigmented melanocytic nevi, large pigmented freckles (*ephelides*) may occur in the skin of some albinos.

especially on the cheeks forehead extensor aspects of the arms neck and the upper region of the back and chest, but not on the legs [14, 26 \*] Exposure to sunlight may be a factor in their development Their clinical and histologic appearance as reported by Barnicot [14] is similar to that of ephelides in normal Caucasians

**Hair Color** The hair color in the Negro albino ranges from "white faintly tinged with yellow to a yellowish brown color" [14] The hair is said to darken with age as in normal persons Microscopic examination of Negro albino hair reveals the presence of fine pigment granules in the cortex in the darker strands of hair [14 28] The hair of Caucasian albinos is often, but not always white and ranges through all shades of cream yellow yellow red and even strong red Thus the range of the hair color of the Caucasian albino merges with that of the normal blond Caucasian Indeed some extreme blond nonalbino hair is lighter than that of the true albino

Banding (alternating white and dark bands) has been observed in the African albino

**Texture** The texture of Caucasian albino hair is unusually fine but that of the Negro albino is unchanged [28]

**Eye Color** There is a common misconception regarding the iris color and pupillary reflex of generalized albinos which may be based on the pink eye of albino mice and rats While it is true that the iris color is a light gray and the pupil red in generalized albino infants the commonest finding in generalized adult albinos is a blue gray translucent iris a black pupil, and a decrease or absence of retinal and choroidal pigment The red pupil occasionally noted in generalized albino infants darkens to black with age

The iris color in Negro albinos ranges from a pale blue to a cinnamon brown [26] A range of light brown green blue green and blue was recorded by Barnicot [14] in African Negro albinos He observed only a single albino child with a red pupil reflex

The fundus is usually bright orange red with prominent choroidal and retinal vessels The macula is uniformly pink Falls [7] believes that a greater degree of pinkness is correlated with more efficient visual acuity The absence or poor development of the fovea centralis in the albino eye may be the chief cause of the defective vision [29]

Perhaps the clinical feature of the albino most obvious to both patient and physician is photophobia present even in the relatively darkly pigmented Negro albino iris The photophobia probably results from the 'dazzling' introduced by the lack of contrast between the retinal impulse and the surrounding media

Visual acuity is almost always decreased in generalized albinism In a study of 16 albinos Edmunds [30] found an average visual acuity of 20/200 Nine were hypermetropic 5 myopic and 13 had astigmatism The

visual fields are abnormal and usually there is a central scotoma. Color vision and dark adaptation are normal. Strabismus (both divergent and convergent type) is not uncommon.

The albino eye almost always shows a horizontal nystagmus. The poor central vision (due to central scotoma) allows the subcortical rhythm center normally held in check by the cortex to produce involuntary rhythmic oscillations [30]. Congenital ocular abnormalities associated with albinism include absence of the sphincter muscle, partial aniridia, congenital pupillary membrane, atrophy of the disk and coloboma of the mesodermal tissue of the iris [31].

The albino eye is normal histologically except for the decrease or absence of pigment in the retinal pigment epithelium and choroid and hypoplasia or aplasia of the macular area.

**Miscellaneous Abnormalities Associated with Generalized Albinism** Numerous abnormalities have been associated with generalized albinism. These include deaf mutism, epilepsy, polydactyly and oligophrenia. The incidence of these abnormalities has not been reported in a large series and the frequency of their occurrence is not known.

The intelligence of generalized albinos is normal [31, 32] but no large series has been studied. The condition is compatible with unusual intelligence. Sachs [33] was an albino scientist who chemically analyzed his own hair. Reverend W. A. Spooner (1844-1930), a distinguished scholar, Warden of New College, Oxford University and of spoonerism fame, was a generalized albino.

**Localized Albinism of the Eye (Ocular Albinism)** There are two types of albinism limited to the eye. In one there is absence of pigment in the retinal pigment epithelium. In the other there is additionally a decreased quantity of iris pigment. Both varieties have nystagmus and decreased visual acuity but normal pigmentation of the skin and hair.

**Localized Albinism of the Skin and Hair (Partial Albinism)** Albinism may be localized to the skin and hair without eye involvement (Fig. 13-4). This has been variously designated as albinoidism, congenital achromia, congenital leukoderma, congenital vitiligo, poikilochromia alba, white spotting and piebaldness. When limited to the hair alone it may be called poliosis circumscripta, white forelock, white blaze and partial albinism. The essential features have been summarized by Cooke [34]. Depigmentation of a tuft of hair in the midfrontal area (white forelock) is present in the majority of cases. The loss of hair color may extend over one-half of the frontal area and may involve the eyebrows and eyelashes without involving the uveal tract or retinal pigment epithelium. The depigmentation may extend to one-half of the face to include the orbital, infraorbital, buccal and zygomatic regions.

Unpigmented skin of the trunk and extremities (which may be unilateral or bilateral) most commonly involve the abdomen and ventral

thorax. The unpigmented areas of the face, trunk, or extremities may simulate the clinical picture of vitiligo.

Unpigmented areas may partially repigment in the second and third decade. A father may have almost completely repigmented areas on the abdomen whereas his son may show large white macular lesions in the same area [35].



Fig. 13-4 Localized cutaneous albinism in two siblings. Note the white forelock. (From H. M. Jahr et al. [12]. By permission of the American Medical Association.)

### BIOCHEMICAL LESION OF HUMAN ALBINISM

Speculations on the nature of albinism were made by Garrod [4] in 1908 when he attributed the absence of melanin to a *metabolic* defect of the pigment cell and in 1911 by Pearson [1] who maintained that albinism was a *structural* defect or an absence of pigment cells. These conflicting views have been partially resolved in the past 50 years.

Much progress has been made in knowledge of the genes in mice and guinea pigs which control the differentiation of pigment cells, the size, shape, and color intensity of the melanin granule and the synthesis of the enzyme tyrosinase. Four factors influence the activity and function of melanocytes in the mouse: (1) the genotype of the melanoblast, (2) the genotype of the environmental cells, (3) the environmental history of the melanoblast, and (4) the differentiated characteristics of the environmental cells [36]. In man it has not been possible to identify the genes responsible for hair, eye, and skin color and to relate the various types of

albinism to specific gene loci but this has been done with some success in other species

It is now possible to give a fairly complete answer to the question of whether generalized albinism in man is a *structural* or a *metabolic* defect. In the last decade techniques have been developed for study of the biochemistry and cytochemistry of melanogenesis and the fine structure of melanocytes. Two basic questions must be answered for an understanding of the nature of albinism: (1) Are melanocytes present in albino skin, hair and eyes? (2) What is the evidence for a biochemical lesion in albinism?

#### EVIDENCE FOR THE PRESENCE OF MELANOCYTES

The identification of amelanotic melanocytes by stains is un satisfactory. In 1952 Becker et al. [37] reported the identification of melanocytes in

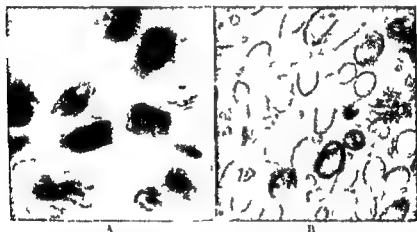


Fig. 13-5. A: High magnification ( $\times 30,000$ ) field of forming melanosomes in a melanocyte from dark Caucasian hair. B: Same field from an albino hair bulb. Granules show varying stages of melanization in (A) and absence of melanization in (B). (From V. A. Barnard et al. [33]. By permission of Academic Press Inc., New York.)

human albino skin by the gold impregnation technique. Silvers [38] has now shown by ingenious studies in mice that gold-impregnated dendritic-shaped cells are present in limb buds removed before the stage of migration of melanocytes from the neural crest into these structures. Thus the branched cells revealed by gold chloride have no relation to melanocytes.

The study of melanocytes with electron microscopy has provided a positive identification of amelanotic melanocytes containing amelanotic melanin granules [39]. The melanocytes in the human albino hair bulb were identified by their cytoplasmic dendritic processes and were shown to contain in addition to mitochondria and Golgi material numerous ovoid or rod-shaped bodies 0.5  $\mu$ m or less in length (Fig. 13-5). These latter

structures represent amelanin melanin granules. These cells presumably have normal biochemical systems with the exception of the enzyme tyrosinase.

The presence of melanocytes in albino skin is also suggested by the occurrence of benign melanocytic nevi and malignant melanoma in generalized albinism (Fig. 13.3).

Finally, pigmentless melanocytes in generalized albinism are easily identified in the retinal pigment epithelium.

On the basis of these facts it seems fairly certain that the structural basis for melanogenesis is present in generalized albinism. There is no evidence which would allow one to consider generalized albinism a structural defect as proposed by Pearson [1].

#### SITE OF THE METABOLIC BLOCK

*The biochemical lesion of albinism consists of a failure of melanocytes to synthesize tyrosinase, a copper-containing oxidase required for the conversion of tyrosine to melanin.*

Tyrosinase is synthesized and localized on the melanin granule, the metabolic unit of melanogenesis. The melanin granule is a spherical or rod-shaped body from 0.1 to 0.5  $\mu$  in diameter in the cytoplasm of the melanocyte. It is distinct from mitochondria. In addition to tyrosinase, melanin granules contain two other enzymes, succinic dehydrogenase and cytochrome oxidase [50].

The process of melanogenesis involves a conversion of tyrosine to a brown or black insoluble polymer (Fig. 13.6). Tyrosinase catalyzes the first step, the hydroxylation of tyrosine to dihydroxyphenylalanine (dopa). The next step, the oxidation of dopa to dopa quinone, is markedly accelerated by tyrosinase but can occur nonenzymatically.

The balance of the pathway may be nonenzymatic. Dopa quinone cyclizes to 5,6-dihydroxyindole-2-carboxylic acid and then is oxidized to the corresponding quinone, dopachrome. Dopachrome by an internal oxidation-reduction loses carbon dioxide to form 5,6-dihydroxyindole, which is the immediate precursor of melanin. The polymerization of the monomer 5,6-dihydroxyindole to a polymer (melanin) occurs by oxidation to indole 5,6-quinone and repeated condensations between the anionoid centers in the pyrrole ring of one molecule and cationoid centers in the quinonoid ring of adjacent molecules. This gives rise to a polymer of high molecular weight [41]. For this polymerization the 3 position and the 4 or 7 position must be free, but the 2, 3, 4, and 7 positions all play a part in the building up of a three-dimensional polymer of high molecular weight [41] (Fig. 13.7). The melanin polymer is attached to protein through its quinone linkages with amino or sulfhydryl groups of protein.

Melanin pigment (in the melanocyte) seen in the light microscope as a

brown or black cytoplasmic inclusion = not an inert particle but a functioning metabolic unit containing a heterogeneous mixture of protein

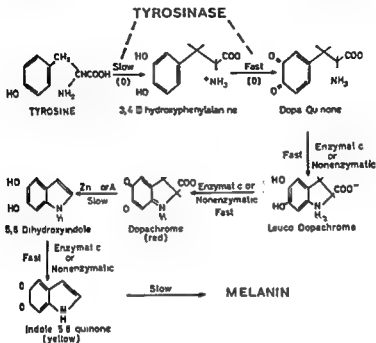


Fig 13-4 Metabolic pathway of tyrosine to melanin

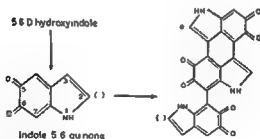


Fig 13-5 Suggested polymerization in melanin formation. Asterisks indicate sites of coupling of monomers (after I I T C o tie d l [11])

melano-protein tyrosinase and perhaps two other enzymes. The elucidation of the structure of natural melanin = a challenging and difficult problem because of the heterogeneity of the melanin granule.

Melanin is defined as a brown or black insoluble pigment contained on melanin granules in the cytoplasm of melanocytes and formed from tyrosine by tyrosinase. The melanin in the cells of the melanocyte system



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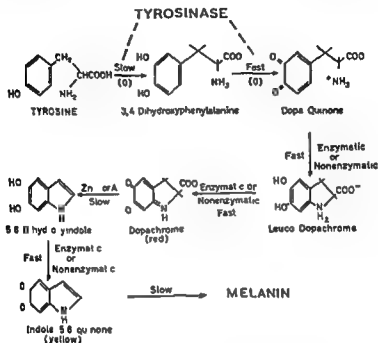


Fig 13-6 Metabolic pathway of tyrosine to melanin

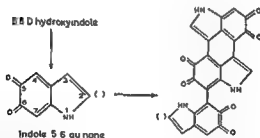


Fig 13-7 Suggested polymerization in melanin formation. Asterisks indicate sites of coupling of monomers (After R I T Crombie et al [41])

melano protein tyrosinase and perhaps two other enzymes. The elucidation of the structure of natural melanin is a challenging and difficult problem because of the heterogeneity of the melanin granule.

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is included in this definition because the melanin pigment is contained in melanocytes and the tyrosine-tyrosinase system occurs in the melanocytes of the skin [42] hair bulb [43] retinal pigment epithelium [6] and uveal tract [40]. Melanocytes in the paraneuroblastoma have not yet been studied for tyrosine-tyrosinase activity, but it is probable that they are part of the melanocyte system because of the origin of primary malignant melanoma in the paraneuroblastoma.

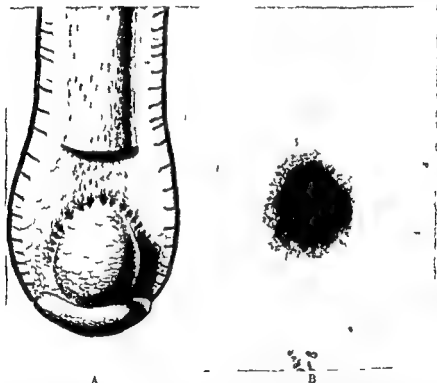


Fig. 13-8. Normal human black hair. A: The melanocytes occupy the upper portion of the hair matrix. B: Radioautographic tyrosinase method. Lithium carmine. Magnification  $\times 300$ . The tyrosinase contained in the hair bulb melanocytes has catalyzed the oxidation of  $C^{14}$ -labeled tyrosine to  $C^{14}$ -labeled melanin. The sites of radioactive melanin are represented by dense masses of silver grains. Tissue was incubated *in vitro* in 0.3 microcurie of DL-tyrosine- $2-C^{14}$ .

The nature of the pigment in the substantia nigra and the locus caeruleus has not been clarified. The pigment in these areas is unchanged in generalized albinism [44] and this would suggest that it is elaborated by some other pathway than by the action of tyrosinase. Malignant melanomas or melanocytic nevi do not arise from the pigmented cells of the substantia nigra and locus caeruleus. However, these data are not

sufficient to conclude definitely that the pigment is not tyrosine-melanin derived from tyrosine by the action of tyrosinase. Further studies are necessary especially to identify tyrosinase in these cells. Fellman [45] has proposed that the pigment in these two areas arises from the oxidative polymerization of epinephrine or norepinephrine.

Manometric measurement of the tyrosinase in certain alleles of the albino series of mice (C series) has revealed a complete absence of tyrosinase activity [46]. In man it is not feasible to obtain large enough quantities of melanocytes from the hair bulb or skin for manometric estimation of tyrosinase.

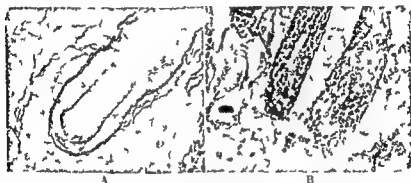


Fig 13-9 Albino hair bulbs of man (A) and mouse (B) genotype (a/a B/B c/c D/D P/P). Radioautographs, tyrosinase method. Compare with Fig 13-8. The virtual absence of silver grains, indicative of the lack of tyrosinase in the melanocytes of the albino hair bulbs of man and mouse genotype.

The use of histochemical procedures for tyrosinase detection has proved highly satisfactory and rewarding. The available methods are more sensitive than manometry, are equally specific, and have the added advantage of localizing precisely the enzyme activity in the melanocytes. Most of the histochemical studies of melanin formation in the past have utilized dopa, the second product in the oxidation of tyrosine to melanin, as substrate. This has serious limitations: for dopa is unstable and is nonspecifically oxidized to melanin by a variety of enzymes and oxidizing systems other than tyrosinase.

The development of radioautography utilizing  $C^{14}$  labeled tyrosine as substrate has provided a relatively simple method for detection of tyrosinase activity [43, 47, 48]. This method, now fairly well standardized, is highly specific and more sensitive than either manometric methods or histochemical procedures with nonradioactive substrates.

Figure 13-11 illustrates the demonstration of tyrosinase with the histochemical radioautographic technique. Tyrosinase cannot be detected in

the melanocytes of the skin or hair bulb of the human generalized albino. The absence of tyrosinase in the hair bulb of the human generalized albino and the albino mouse of specific genotype is shown in Fig. 13.9.

Tyrosinase activity in the human epidermal melanocyte is of such low concentration (or is present in an inhibited form) that it cannot be demonstrated unless the skin is previously irradiated *in vivo* [42]. When ultraviolet irradiated skin slices are incubated in tyrosine melanin readily appears in the melanocyte as brown granules in the perikaryon and cytoplasmic processes.

The failure of melanin formation from the melanogenic substrates tyrosine and dopa in albino melanocytes could hypothetically result from a lack of machinery in the melanocyte (1) for tyrosinase synthesis (2) for melanin polymerization or (3) for synthesis of the precise protein of the

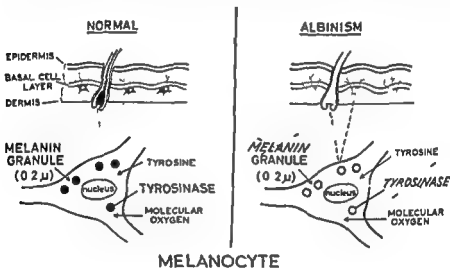


Fig. 13.10 The biochemical lesion in albinism

melanin granule. Since amelanotic pigment granules in the human albino hair bulb appear identical to the melanotic pigment granules of the normal hair bulb when viewed in the electron microscope it would appear that the metabolic defect in albinism is a failure either of tyrosinase synthesis or of melanin polymerization. The latter possibility is precluded by the absence of oxygen consumption in preparations of fetal albino guinea pig skin in the presence of tyrosine [49]. This indicates a failure of hydroxylation and dehydrogenation. If the defect in albinism were simply a failure of the monomer indole 5,6 quinone to polymerize to melanin oxygen consumption as required for the formation of the monomer would be unimpaired. Thus from all the available evidence it can be concluded that the biochemical lesion in albinism is an absence of the gene in the

melanocyte which is required for the synthesis of tyrosine use in the melanin granule (Fig 13 10)

### EFFECT OF THE ABSENCE OF TYROSINASE ON AROMATIC AMINO ACID METABOLISM

In phenylketonuria where decreased melanin pigmentation of the hair [50] and iris [51] is found there is a marked accumulation of phenylalanine and related metabolites in the extracellular fluids as a result of the reduced amount or complete absence of L phenylalanine oxidase [52] (cf Chap 10)

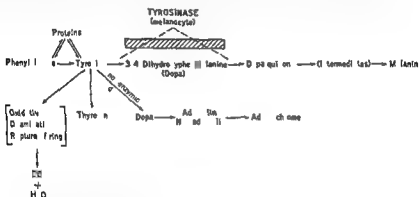


Fig 13-11 Intermediary metabolism of phenylalanine and tyrosine and site of metabolic block in albinism

The accumulated phenylalanine and its metabolites perhaps acting as competitive inhibitors cause (1) inhibition of tyrosinase [53 54] resulting in a decrease in hair and iris color (2) inhibition of the incorporation of tyrosine into proteins of liver slices [55] (3) an accumulation of phenylalanine and tyrosine in the adrenal gland and reduced amounts of norepinephrine and epinephrine [45] and (4) a decreased amount of pigment in the substantia nigra and locus caeruleus [46]. A parallel effect of accumulated metabolites in albinism on the other aromatic pathways may be found but this problem has not been studied. It will be necessary first to determine the concentration of tyrosine and related metabolites in the extracellular fluids of albinos. The content of pressor amines in the urine of a generalized albino was within normal limits [56]. It is probable that tyrosine is hydroxylated to dopa in the adrenal gland by another pathway not requiring tyrosinase. This would explain the normal amounts of pressor amines in albinos who lack tyrosinase in melanocytes and cannot hydroxylate tyrosine to dopa in these cells (Fig 13 11).

In order to conclude that a particular metabolic disorder is a consequence of a deficiency of a certain enzyme two types of evidence are required [57]

1 An *in vitro* demonstration of a decrease or absence of the enzyme with certainty that the failure to detect the enzyme is not due to the presence of inhibitors or some other environmental factor

2 Demonstration of biochemical changes in body fluids and tissues which can be attributed to the failure of a particular reaction normally catalyzed by the enzyme

The biochemical and histochemical studies described in this review appear to satisfy the first requirement of the *in vitro* demonstration of an absence of tyrosinase in the melanocytes. The second postulate is satisfied by the decrease or absence of melanin formation in the skin, hair, and eyes which is the direct result of the absence of tyrosinase which catalyzes the tyrosine-melanin pathway.

## GENETICS

Both universal and generalized albinism are inherited as autosomal recessive traits. This interpretation is based on more than 700 pedigrees reported in the past 30 years. Hogben's [58] analysis of Pearson's data consisting of over 600 pedigrees is the most complete available. Pipkin and Pipkin [20] in a study of the pedigrees of 5 albino families concluded that 3 were autosomal recessive and 2 autosomal dominant. An excellent recent study of 122 albinos resident in Northern Ireland [2] supports an autosomal recessive inheritance. In Froggatt's study [2] the consanguinity rate for first cousin matings was 4.49 per cent. Higher consanguinity rates have been reported by Pearson [1] (11 to 14 per cent) and Sanders [59] (10.7 per cent). A loose linkage of the genes for albinism and sickle cell anemia is suggested by the observation of a Negro family in which albinism and sickle cell anemia occurred [60]. One albino was homozygous for the sickle cell gene. Wright (quoted by Castle [61]) suggested that albinism in man may be inherited as a graded series of allelomorphs similar to the guinea pig series. Knox has commented on the occurrence of albinism on the basis of a series of allelic as well as nonallelic genes. He cites as evidence the instance of two normally pigmented infants with good vision born from the union of two generalized albinos [64]. Knox also cites the existence of *rufous* albinism or "xanthism" as suggestive of allelic genes. Although xanthism has not been directly linked with generalized albinism, Loewenthal [63] has reported the occurrence of nystagmus and freckling (two characteristic features of generalized albinism) in two African negroes with xanthism. A discussion of the genetic history of albinism is included in Knox's [64] excellent review.

Localized albinism of the skin (partial albinism) is inherited as a dominant gene with high penetrance [12, 35, 59]. In a complete summary of the literature, Cooke [34] collected 23 families. She found that when one parent had the trait, more than half (53.2 per cent) of the 769 children

were affected while of more than 474 children born of unaffected parents only 15 (3.1 per cent) inherited the trait. The various discrepancies in the transmission of localized cutaneous albinism may be explained by lack of recognition of the trait because of fair skins in those subjects in whom the characteristic white forelock is absent. In addition localized cutaneous albinism is easily confused with vitiligo localized to one area.

Localized cutaneous albinism has been known to occur in the same pedigree as generalized albinism. While the two conditions are presumably not related to each other they are both genetically transmitted.

Localized pigmentary loss in the hair of mice (known as spotting) is related to the action of genes at seven different loci. In this anomaly there is a loss of melanocytes from the hair follicles of the spotted regions. The pigmentary defect is not the result of gene-enzyme deficiency as in albinism [65]. Since there have been no studies of localized (partial) albinism to determine the presence or absence of melanocytes one cannot state for sure whether localized albinism in man is a metabolic (tyrosinase) defect or a structural (melanocyte) defect.

Localized albinism of the eye is inherited as an X chromosomal (sex linked) trait. Waardenburg [66] observed iris translucency in the heterozygote or carrier female and Falls [67] described a characteristic fundus in the female heterozygote. This pigmentary change consists of small clots of coarse cocoa brown pigment clusters and stippling of the macular areas. The subjects have normal vision. Thus Waardenburg's and Falls' observations suggest an intermediate inheritance pattern in which there is limited expression in the heterozygous condition.

## SUMMARY

1. Albinism is an inherited metabolic defect of the melanocyte in which that cell fails to form melanin. The metabolic defect may involve the entire melanocyte system (universal albinism), the majority of the melanocytes in the melanocyte system (generalized albinism) or only one locus of melanocyte (localized albinism of the skin or eye).

2. Universal albinism has been observed in almost all species. Generalized albinism in man occurs in a ratio of from 1 in 1000 to 1 in 2000.

3. Pigmentless melanocytes are present in the albino. Amelanin melanin granules can be visualized in the albino hair bulb with the aid of the electron microscope.

4. The biochemical lesion in albinism is a failure of the melanocyte to synthesize tyrosinase. This enzyme is required for melanin formation in the melanocyte. The enzyme defect has been established by a histochemical radioautographic technique utilizing  $C^{14}$  labeled tyrosine as substrate.

5. The absence of tyrosinase in the melanocyte system may have an



effect on other aromatic amino acid pathways but thus far there has been little or no investigation of possible accumulated metabolites in albinism. The formation of epinephrine and norepinephrine does not appear to be affected in the albino.

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## Chapter 14

### Primary Hyperoxaluria and Oxalosis\*

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*James B Wyngaarden and T David Lider*

#### DEFINITIONS

*Primary hyperoxaluria* is a rare disorder characterized clinically by progressive bilateral calcium oxalate urolithiasis and nephrocalcinosis beginning in early childhood and by death in childhood or early adult life from renal failure. Recurrent infection of the urinary tract and hypertension are common secondary features. The disorder is characterized biochemically by a continuous high urinary oxalate excretion which is thought to result from a genetically controlled metabolic defect leading to overproduction of oxalate. The additional diagnosis *oxalosis* signifies the presence of disseminated extrarenal calcium oxalate deposits which may develop as a stage in the natural history of primary hyperoxaluria.

#### HISTORICAL INTRODUCTION

Calcium oxalate crystals were first identified in urine by Donné in 1838. In the following years the medical literature accumulated many reports in which attempts were made to correlate diverse clinical signs and symptoms with the excretion of oxalate crystals. This endeavor gradually declined when it was appreciated that calcium oxalate crystals are normal constituents of urine whose formation is dependent upon the pH of the urine and the concentration of other solutes. A voluminous and often uncritical literature dealing with oxalate metabolism was extensively reviewed in 1945 by Jeghers and Murphy [1].

The first reported case of oxalosis is probably that of Lepoutre [2] who in 1925 briefly described a 4½ year-old child with bilateral urolithiasis in whom a renal biopsy showed extensive crystalline deposits of

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calcium oxalate. In 1950, Davis, Klingberg and Stowell [3] reported the case of a 12 year-old boy in whom renal calculus formation, nephrocalcinosis, and deposition of calcium oxalate in extrarenal tissues were demonstrated. They suggested the possibility of an underlying metabolic defect. In 1952, Chou and Donohue [4] published a similar case and first employed the term 'oxalosis'. The first recorded observation of hyperoxaluria in a patient with probable oxalosis is that of Newns and Black (1953) [5] but histologic demonstration of oxalate deposits in tissues was not obtained. In 1954, Aponte and Letter [6] described three cases of oxalate nephrocalcinosis occurring in one family. All were boys and the first two were identical twins. Death was due to renal failure at 16  $13\frac{1}{2}$  and 9 years respectively. The twins had urinary oxalate excretions of 200 and 180 mg per 24 hr and both showed oxalate deposits in vertebral marrow at autopsy. The other two cases are the first definite examples of oxalosis in which all critical criteria are fulfilled.

## CLINICAL REVIEW

About thirty case reports have been published which suggest the diagnosis of oxalosis, but only 19 of these may be considered reasonably definite examples on the basis of early age of onset and demonstration of hyperoxaluria or extrarenal deposits of calcium oxalate or both [3-15, 17-19]. The major features of these 19 cases and of one new case are summarized in Table 14-1.

The age at onset ranged from 15 months to 17 years, but the majority of patients at onset were in the 1 to 4 year range. The majority of the patients were males and all subjects in whom race was recorded were white. In 16 fatal cases the duration of the illness from onset of symptoms ranged from 1 month to 18 years with a mean of 4 years.

Although the disease is generally characterized by repeated attacks of renal colic, hematuria and passage of calculi, 4 cases presented in uremia without this history [6, 7]. The diagnosis was established prior to death in 5 cases [5, 11, 17, 19]. In each, multiple pure calcium oxalate stones were passed; many calcium oxalate crystals were found in the urine and the 24 hr urinary excretion of oxalate was elevated. Nephrocalcinosis was diagnosed by x-ray in 3 of the 5 cases and by biopsy in a fourth. Oxalosis was established in 1 by examination of bone marrow [19].

## PHYSICAL FINDINGS

Physical examination reveals nothing characteristic of this disease. In spite of chronic renal disease, the blood pressure is frequently normal.

The eyes have been examined carefully for calcium oxalate crystals in at least three cases [11, 17] but thus far none has been found although they occur in other disorders [20, 21].

The kidneys have not been palpably enlarged and no soft tissue calcifications have been described.

#### LABORATORY AND X RAY FINDINGS

A plain x ray film of the abdomen will usually reveal the presence of dense bilateral renal calculi which may frequently be recognized as calcium oxalate stones by their characteristic crystalline structure with spicules radiating from a central point. They are usually triangular or jackstone in shape [22]. Stones are also frequently observed in the ureters and bladder.

As the disease progresses small focal calcifications appear in the area of the kidney parenchyma opposite the second and third lumbar vertebrae. Radiographic signs of nephrocalcinosis are usually limited to the medullary regions but in two cases [8, 10] the entire kidney was diffusely involved. There was also extensive calcification of ureters and renal pelves in one case reported by Aponte and Letter [6].

Skeletal x rays were normal in 7 of 9 patients. In 2 [8, 9] changes consistent with renal osteodystrophy were found. Similar x rays were published by Caffey [22a] from a patient with oxalosis whose case has not been described in detail.

Serum calcium levels above 10.5 mg per 100 ml were reported in 8 cases but remained consistently elevated in only 3 [3, 11]. In each of these instances 24 hr urinary calcium excretion was normal or low. The serum phosphorus level was normal in all determinations until renal damage resulted in phosphorus retention. Serum alkaline phosphatase level was also normal. There is little reason to implicate parathyroid hyperfunction in these patients except as a secondary feature accompanying renal failure.

The urine frequently showed pyuria, hematuria and proteinuria and calcium oxalate crystals were often noted. Urinary amino acid excretion was normal in 4 patients [5, 9, 11]. Cystine was not increased in the urine of 8 subjects in whom it was measured [5, 6].

#### PATHOLOGY

On gross examination the kidneys are usually small with thickened capsules which strip with difficulty. The surfaces are granular and contain focal depressed scars. They are usually cut with noticeably increased resistance and in most cases a gritty sensation is present as though one were cutting through sand. On the cut surface the cortex is thin and the pelves frequently contain calculi. Small crystals may sometimes be seen in the parenchyma with the naked eye.

On microscopic examination interstitial fibrosis and interstitial nephritis may be present. Refractile crystals of various sizes are seen in the renal

tubules which may have compressed or destroyed the tubular epithelium (Fig 14 1) The crystals may extend into the interstitial spaces and have been described in the tunica media and adventitia of small renal arteries and arterioles The glomeruli are usually normal except for some moderate pericapsular fibrosis and a rare hyalinized glomerulus Extrarenal deposits

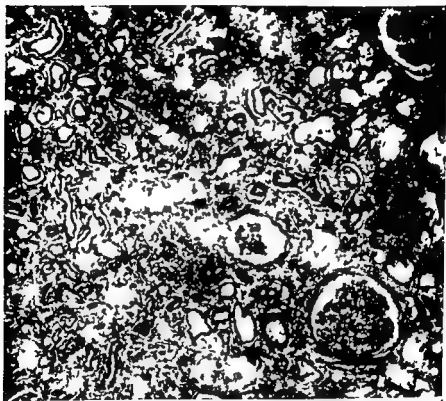


Fig 14 1 Oxalate nephrocalcinosis from a patient with primary hyperoxaluria and oxalosis Most of the calcium oxalate crystals are related to the remains of renal tubules (Half-crossed Nicol prisms  $\times 70$ ) (From E F Scowen et al [15] with permission of the authors and publisher)

of crystals have frequently been described in the bone marrow (Fig 14 2) cartilage (Fig 14 3) myocardium thyroid spleen liver lungs thymus pituitary adrenal testes pancreas parathyroids lymph nodes, and in the walls of veins arteries and arterioles [5-19]

Microscopically the crystals appear round and globular or rhomboidal in shape and have a radial rosette like pattern They have a slightly yellowish tinge and are doubly refractile under polarized light They do not stain with hematoxylin and eosin In most cases they do not stain with special techniques such as that of von Kossa The few instances in which the crystals did stain black with von Kossa's stain [9-11 16]

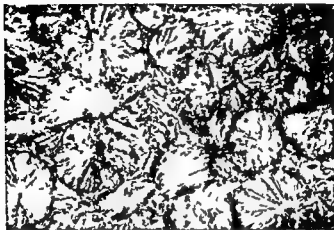


Fig 14-2 Calcium oxalate crystals in vertebral bone marrow from a patient with oxalosis ( $\times 150$ ) (From H O Dunn [9] with permission of the author and publisher)



Fig 14-3 Calcium oxalate crystals in costochondral cartilage of a patient with primary hyperoxaluria and oxalosis visualized under partial polarization ( $\times 175$ ) (From J T Godwin et al [17] with permission of the author and publisher)

may have been due to the presence of calcium carbonate or phosphate which was coprecipitated with oxalate [8]

The crystals may be identified in histologic sections as calcium oxalate by various chemical techniques. The crystals are soluble when unstained deparaffinized sections are treated with concentrated hydrochloric or sulfuric acid and insoluble with lithium carbonate glacial acetic acid



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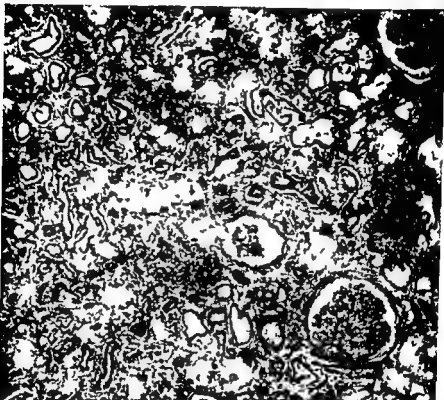


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Oxalic acid is a relatively strong acid

$$K = 6.5 \times 10^{-2}$$

$$K = 6.1 \times 10^{-3}$$

It forms both acid and neutral salts; mono and die ters a monoamide known as oxamic acid and a diamide known as oxamide

Oxalic acid is easily oxidized by ferric compounds and potassium permanganate and the latter compound is often used in quantitative determinations of oxalate by titration according to the following reaction



In neutral or alkaline pH oxalate forms a calcium salt of very low solubility. The precipitation of calcium oxalate from solution is inhibited by the presence of urea, various ions such as sulfate and magnesium [27-29] and probably certain colloids [30]. Under physiologic conditions urine is frequently supersaturated with calcium oxalate [27-31].

## BIOSYNTHESIS OF OXALIC ACID IN MAMMALIAN SYSTEMS

In mammalian systems the only known direct precursor of oxalic acid is glyoxylic acid. Glyoxylic acid can arise from several sources; the best known of which is glycine

### GLYCINE PATHWAY

Although the glycine pathway was postulated by Dakin as early as 1922 [31] the first demonstration that glycine serves as a precursor of oxalic acid was by Ratner, Nocito and Green in 1944 [32]. They prepared from a variety of mammalian kidney and liver sources an enzyme which oxidizes glycine (or sarcosine) to glyoxylic acid in the presence of flavin adenine dinucleotide (FAD). They named this enzyme glycine oxidase. They also found an enzyme system in rat kidney and liver and cat muscle which catalyzes the conversion of glyoxylic acid to oxalic acid. They believed this enzyme to be a mutase of the general type



Although milk xanthine oxidase (aldehyde oxidase) also catalyzed the oxidation of glyoxylate to oxalate, the presence of a second system in these tissues was indicated by the finding that the reaction proceeded anaerobically. Kidney tissue appears to be the richest source of both glycine oxidase and mutase.

A second reaction by which glycine may be converted to glyoxylic acid is that of transamination between glycine and  $\alpha$  ketoglutaric acid [33]. This reaction is freely reversible.

Weinhouse and Friedmann [34] showed that glycine  $\text{C}^{14}$  administered intraperitoneally in rats was extensively converted to glyoxylic acid and

concentrated ammonium hydroxide or concentrated potassium hydroxide. When the crystals are dissolved in concentrated sulfuric acid, small needle-like crystals of calcium sulfate may occasionally form [16]. If paraffin sections of tissue are first incinerated at 450°C for 30 min, calcium oxalate is oxidized to the carbonate and small bubbles of carbon dioxide may be seen microscopically when concentrated sulfuric acid is allowed to run under the cover slip [23].

The crystals may also be identified as the monohydrate of calcium oxalate by optical examination. The average width of single-needle crystals is 0.005 mm and the average length is 0.05 mm. They have a birefringence of about 0.15 and an extinction angle of approximately 30° with respect to the length of the crystal. The  $\alpha$  and  $\gamma$  indices of refraction are 1.49 and 1.65 by the immersion method. The crystals have also been identified by x-ray diffraction in several cases [9, 10, 16, 17, 19].

Pathologically, the lesions are similar to those produced by experimental oxalate poisoning in animals in which the renal tubules are filled with calcium oxalate crystals compressing and causing necrosis of tubular epithelium [21-26].

### TISSUE OXALATE ANALYSES

Selected tissues were collected post mortem from a case of primary hyperoxaluria by Scowen, Stansfield, and Watts [18] and analyzed for calcium and oxalate contents. The kidneys contained 67.2 and 65.0 mg  $(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$  per gm dry weight; the heart 1.19 mg  $(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$  per gm dry weight. Control tissue from four patients dying of unrelated illnesses contained from 0 to 0.06 mg  $(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$  per gm dry weight. Oxalate values of skeletal muscle and liver were not elevated in the hyperoxaluric subject and were 0.03 to 0.04 mg  $(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$  per gm dry weight, respectively. Calcium values were correspondingly high in kidney and heart and were not appreciably elevated in skeletal muscle and liver.

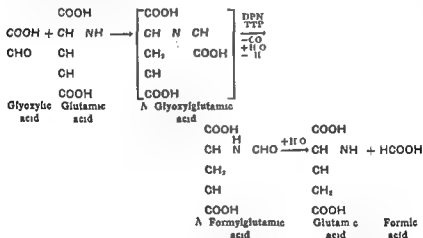
High concentrations of oxalate were present in the cerebrospinal and pleural fluids collected post mortem from the patient with primary hyperoxaluria. No oxalate was detected in the cerebrospinal fluid from any of five 'control' subjects including one who died from uremia due to malignant hypertension.

### OXALIC ACID

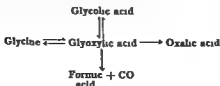
Oxalic acid is a dicarboxylic acid,  $\text{HOOC}-\text{COOH}$ , and may be crystallized as the white dihydrate. The hydrate loses its water at about 100°C and the anhydrous acid sublimes with decomposition above 150°C. It is soluble to the extent of 8.7 gm per 100 gm water at 20°C.

## Glyoxylic acid

In addition to its reduction to glycolic acid its transamination to glycine and its direct oxidation to oxalate glyoxylate may be cleaved oxidatively to yield  $\text{CO}_2$  and formic acid Nakada and Sund [39] have described an enzyme system tentatively designated glyoxylic acid dehydrogenase from rat liver mitochondria which requires diphosphopyridine nucleotide (DPN) thiamine pyrophosphate (TTP) and manganese ( $\text{Mn}^{++}$ ) plus a stoichiometric quantity of L-glutamic acid Because of the requirement for glutamic acid and the isolation of the intermediate *N* formylglutamic acid they suggested that the process involves an initial condensation of glyoxylic acid and L-glutamic acid to yield a hypothetic intermediate *N* glyoxylglutamic acid which is then converted to  $\text{CO}_2$  and *N* formylglutamic acid by the TTP DPN-dependent enzyme glyoxylic acid dehydrogenase The formyl derivative may then enter transformylation reactions or proceed by enzymatic hydrolysis to glutamic and formic acids

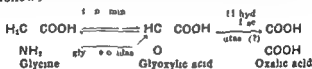


The data of Nakada and Weinhouse suggest that the major fate of glyoxylic acid is normally transamination to glycine a function that requires pyridoxal phosphate as coenzyme Reactions involving glyoxylic acid and oxalate formation are summarized below



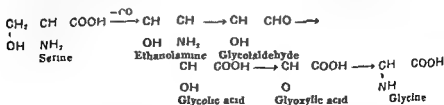
carbon dioxide and sparingly converted to oxalic acid. Glyoxylic acid  $C^{14}$  was extensively converted to glycine (isolated as hippuric acid) and oxalic acid but only sparingly oxidized to  $CO_2$ . Administration of formate and acetate did not result in labeling of oxalate. Nakada and Weinhouse [35] studied glycine oxidation in rat liver and kidney homogenates and slices. They showed that glycine- $2\text{ }C^{14}$  rapidly labeled carbon dioxide and hippurate but not oxalate. Glycolate- $2\text{ }C^{14}$  or glyoxylate- $1,2\text{ }C^{14}$  rapidly labeled all three compounds and oxalate most extensively. This suggested to them that glyoxylate was appreciably converted to oxalate only at high substrate concentrations. When they incubated glycine- $C^{14}$  with pigeon liver homogenates in the presence of a large pool of unlabeled glyoxylate, the glyoxylate became highly labeled and significant incorporation of  $C^{14}$  into oxalate also resulted. When glyoxylate was present in concentrations of 0.001 M or less, it was oxidized to  $CO_2$  and yielded little or no oxalate, but as glyoxylate concentration was increased, oxalate labeling was increased and at a glyoxylate concentration of 0.01 M, oxalate was almost as highly labeled as  $CO_2$ . Since pigeon liver is devoid of xanthine oxidase activity, these results confirm the existence of a second enzyme system capable of oxidizing glyoxylate to oxalate.

The reactions by which oxalate may arise from glycine may be summarized as follows:



#### GLYCOLIC ACID PATHWAY

Glyoxylic acid may be formed in mammalian tissue from precursors other than glycine via glycolic acid. The chief sequence in which glycolic acid occurs is in the metabolism of ethanolamine, formed by decarboxylation of serine [36-38]:



Glyoxylic acid may be reduced to glycolic acid with lactic dehydrogenase and DPNH [35]. The reverse reaction is catalyzed by a flavo-protein, glycolic oxidase [36], and its operation is demonstrated by the conversion of glycolic acid to glyoxylic acid, glycine and oxalic acid in the experiments of Nakada and Weinhouse discussed above.

Large quantities of ingested oxalate may significantly depress calcium absorption from the gastrointestinal tract by forming the insoluble calcium oxalate salt [45-48] The effect of calcium intake upon oxalate absorption has not yet been studied

Archer et al [49] have presented data on the absorption of soluble and insoluble oxalates in 6 normal subjects On a diet with a mean oxalate content of 1 288 mg and a mean calcium content of 976 mg per 24 hr the urinary oxalate excretion ranged from 10 to 34 mg per 24 hr

Four subjects were then fed a solution of sodium oxalate in increasing quantities in four divided doses immediately after meals for six consecutive days The daily dose ranged from 1 0 to 4 0 gm expressed as oxalic acid dihydrate Urinary excretion of oxalate gradually increased over the 6 day period but the mean increases accounted for only 2 3 to 4 5 per cent of the administered dose Some subjects did not show an excretion of oxalate above the normal range until oxalate intake was increased at least 2 5 fold over the basal dietary level

In an identical experiment 2 subjects were fed increasing doses of calcium oxalate over a 6 day period The doses ranged from 1 3 to 4 3 gm as oxalic acid dihydrate The data show a slight increase in oxalate excretion but this is not significantly outside the normal range and not at all comparable to the increase observed during ingestion of soluble sodium oxalate

Dempsey studied 3 normal subjects on a metabolic ward and found that the ingestion of a diet rich in oxalate caused an increase in the 24 hr urinary excretion of oxalate two to four times that observed on a low oxalate diet [50] Instances of oxalate poisoning following rhubarb glut tony [1] or experimental feeding in animals have been recorded [51]

#### OXALATE METABOLISM

Dakin suggested in 1922 that oxalate is an end product of metabolism and is probably not oxidized by mammalian tissues nor essential for any metabolic process [13] This has been substantiated by the finding that isotopically labeled oxalate given subcutaneously or intraperitoneally to rats is excreted unchanged in urine and feces although a small amount is stored in bone and muscle following large doses There is essentially no labeling of respiratory carbon dioxide or urinary hippurate [31 52 53] In human beings also labeled oxalic acid  $C^{14}$  given orally or intravenously does not label expired carbon dioxide and intravenously administered oxalic acid is almost quantitatively recovered in the urine [54]

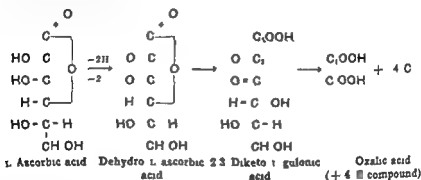
#### OXALATE EXCRETION

The daily excretion of oxalate in urine has been determined by several techniques [49 55-59] it ranges from 9 0 to 56 0 mg as oxalic acid di

## ASCORBIC ACID PATHWAY

L-Ascorbic acid serves as a precursor of urinary oxalate in both animals and man but the specific mechanisms involved have not been elucidated. Following intraperitoneal injection of labeled L-ascorbic acid in guinea pigs urinary oxalate was significantly labeled. When  $C^{14}$  was present only in the carboxyl group of the vitamin, approximately 20 per cent of urinary  $C^{14}$  appeared as oxalic acid in 48 hr, whereas a much smaller percentage was found in oxalic acid when uniformly labeled ascorbic acid was employed [40].

When 30 mg L-ascorbic acid  $C^{14}$  was given intravenously to three human subjects, 10 per cent of the  $C^{14}$  appeared in urine in 24 hr and 70 per cent in 50 days. By far the largest quantity of urinary activity was present in oxalic acid which accounted for 44 per cent of the excreted label. Hellman and Burns [41, 42] have suggested as a partial pathway, the following sequence:



It is not known whether this pathway involves intermediary formation of glyoxylic acid.

## Other Pathways

Other pathways for the biosynthesis of oxalic acid have been found in microbial organisms and are discussed in a recent review by Jakoby and Bhat [43]. The precursors identified are oxaloacetic acid, oxalosuccinic acid, and  $\beta$ -ketoadipic acid.

## OXALATE METABOLISM IN NORMAL MAN

## ABSORPTION

Until recently the major source of oxalate in the body was thought to be ingested foodstuffs, particularly beets, spinach, rhubarb, chocolate, cocoa, and tea. The recent data of Andrews and Viseer [44] may be consulted for the oxalate content of various foods.

urinary oxalate may be derived from ascorbic acid. However, since only about 15 per cent of ascorbic acid carbon appears as oxalate carbon, the average dietary intake of ascorbic acid would not account for more than a few milligrams of urinary oxalate each day.

#### THE MISCIBLE POOL OF OXALATE

The quantity of oxalate present in the body in rapidly miscible form has been determined in 3 normal subjects by isotope dilution techniques [63]. Each subject was given 1 microcurie oxalic acid  $1.2 \text{ C}^{14}$  intravenously, following which the specific activity of urinary oxalate was determined in 2 to 4 hr specimens for 48 hr. The miscible pool and the rate of its turnover were calculated from the linear portion of the specific activity decay curve.

In these 3 subjects the miscible pool of oxalic acid ranged from  $4.7 \pm 0.7$  to  $8.6 \pm 1.1 \text{ mg}$ . The turnover of the pool was rapid, and half times of 2.2 to 2.8 hr were calculated. The daily turnover of oxalate ranged from  $34.0 \pm 6.7$  to  $68.8 \pm 11.3 \text{ mg}$ ; in each case the turnover value approximated (and included within the range of  $1 \pm 2 \text{ s.d.}$ ) the daily excretions of oxalate as determined by the method of Archer et al. [49]. From 89 to 97 per cent of the infused tracer was recovered unchanged in the urine in 36 hr.

#### OXALATE IN BODY FLUIDS

Many procedures have been devised to measure oxalate content of blood directly; they have produced values ranging from 0.23 to 7.5 mg per 100 ml [64-70]. Barrett evaluated the results obtained by several techniques and concluded that normal blood oxalate was approximately 0.5 mg per 100 ml, but that no available method was highly specific or accurate [69]. Verification will have to await a more sensitive, accurate, and specific method of determination.

### METABOLIC STUDIES IN PRIMARY HYPEROXALURIA AND OXALOSIS

#### SERUM OXALATE CONCENTRATION

Serum oxalate determinations have been reported in only one patient with oxalosis. Marshall and Horwith [19] found values of 2.0 and 3.5 mg per 100 ml, compared with a value of 1.45 mg per 100 ml on pooled normal serum.

#### OXALATE EXCRETION

The urinary excretion of oxalate in proved cases of oxalosis has ranged from 33 to 370 mg per day (Table 14.1). Oxalate to creatinine ratios have ranged up to 0.193 mg  $(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$  per mg creatinine [normal—0.02 mg  $(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$  per mg creatinine] [11]. In one patient oxalate



hydrate. By the method of Archer et al [49] the normal urinary oxalate excretion ranges from 90 to 450 mg per day on an average diet.

In three subjects no consistent diurnal variation was observed between successive 8 hr specimens and excretion of oxalate was not significantly correlated with urine volume [54].

In normal subjects the urinary excretion of oxalate was not affected by ingestion of glycine in quantities ranging from 25 gm as a single dose [54] to 100 gm in divided doses daily for 6 days [12]. In 3 subjects the 24 hr excretion of oxalate was doubled following ingestion of 40 gm gelatin which contains 25 per cent glycine [50]. Attempts to reduce oxalate excretion by the daily ingestion of 4 to 40 gm sodium benzoate were unsuccessful in 2 subjects [12]. Administration of 10 to 20 mg pyridoxine hydrochloride to mentally defective children resulted in a 25 to 58 per cent decrease in the 8 hr overnight excretion of oxalate [50]. In 3 normal adult males ingestion of 20 mg pyridoxine hydrochloride was followed by a mean reduction of 29.3 per cent in overnight excretion of oxalate [54]. These reductions may represent an effect on transamination reactions for which pyridoxal 5 phosphate serves as a cofactor [61] diverting glycine or serine or both from conversion to oxalate. After massive dose of ascorbic acid urinary oxalate excretion may be increased. In two studies ingestion of 9 gm ascorbic acid daily for 2 days led to increases in mean oxalate excretion from 41 to 109 mg per day [62] and from 20 to 40 mg per day [54] in 11 and 3 normal subjects respectively.

The mechanism by which the kidneys excrete oxalate is unknown.

#### BIOSYNTHESIS OF OXALATE IN MAN

Following oral administration of glycine  $1\text{ C}^{14}$  or  $1\text{ C}^{13}$  [63, 63a] to normal subjects urinary oxalate is highly labeled within a few hours. When glycine  $1\text{ C}^{13}$  was fed repetitively to 1 control subject until urinary products achieved constancy of isotopic enrichment oxalate was approximately 40 per cent as highly labeled as free glycine isolated from the same samples [63a]. These results establish glycine as a precursor of perhaps 40 per cent of the urinary oxalate in this subject and suggest that the biosynthetic pathway is relatively direct and involves little dilution in pools of intermediates. The most direct pathway would be glycine  $\rightarrow$  glyoxylic acid  $\rightarrow$  oxalic acid.

In 5 controls an average of 0.051 per cent (range 0.029 to 0.081 per cent) of a single oral dose of glycine  $1\text{ C}^{14}$  appeared in urinary oxalate. Since their mean urinary excretion of oxalate (35 mg per day) approximates 0.035 to 0.07 per cent of the daily turnover of the glycine pool (0.5 to 1.0 gm per kg [64]) the incorporation values cited above suggest that a large portion of urinary oxalate may have been derived from glycine in these subjects.

Hellman and Burns [41] have postulated that the major portion of

excretion declined terminally to low levels (3 to 20 mg per day) but the urinary oxalate to creatinine ratio remained high [0.08 to 0.10 mg  $(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$  per mg creatinine] [13]

Archer et al [12] have conducted a series of metabolic studies on 2 patients with primary hyperoxaluria and oxalosis in which they evaluated the effect on urinary oxalate excretion of the ingestion of oxalates and of measures designed indirectly to alter body glycine stores

#### *Oxalate Ingestion*

Following ingestion of sodium oxalate and of rhubarb juice [containing about 4 gm of  $(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$  per liter] both hyperoxaluric subjects showed small increases in urinary oxalate. The increments corresponded to 1.2 to 5.4 per cent of the ingested quantities and thus agreed closely with values obtained earlier following ingestion of soluble oxalate in normal subjects. Calcium oxalate ingestion did not materially alter the level of urinary oxalate.

#### *Dietary Protein Restrictions*

One of two patients showed a small but statistically significant decrease in urinary oxalate excretion during protein restriction, whereas the second patient showed no significant change despite a greater restriction of dietary protein.

#### *Sodium Benzoate Administration*

Sodium benzoate administration (10 to 40 gm per day) was associated with a transient decrease in urinary oxalate excretion in both hyperoxaluric patients. In the first the mean urinary oxalate excretion decreased approximately from 200 to 165 mg per day; in the second approximately from 95 to 65 mg per day. In the latter patient a restricted protein intake did not enhance the response to sodium benzoate.

Prolonged sodium benzoate administration (20 gm per day) was unsuccessful in achieving a permanent reduction in oxalate excretion in one patient. After about 4 weeks the excretion of oxalate gradually returned to high levels.

#### *Glycine Administration*

In one patient the administration of 18 gm glycine per day for 8 days was associated with a slight rise in average oxalate excretion, approximately from 135 to 165 mg per day. Marshall and Horwith [19] report that oxalate excretion was increased following oral ingestion of glycine, glycolic acid, and ascorbic acid in their subject.

#### TRACER STUDIES WITH LABELED GLYCINE

Scowen, Crawhall, and Watts [21, 71a] administered glycine- $1\text{-C}^{14}$  orally every 6 hr for 4 days to patients with primary hyperoxaluria and

TABLE 14 I SUMMARY OF PROVED CASES OF PRIMARY HYPERCALCAEMIA AND OXALOSIS

| Author  | Ref  | Year | Sex | Age at onset | Age at death | Site of metastases | Ca <sup>++</sup> mg (COOH) 24 hr | Period of post mortem | Examination of post mortem |
|---|------|------|-----|--------------|--------------|--------------------|----------------------------------|-----------------------|----------------------------|
| Davis, L. L., Gleason, D. J., Zolner, and R. M. | [15] | 1950 | M   | 1            | 3            | 12                 |                                  | +                     | +                          |
| Nelson, D. R., and R. M.                        | [16] | 1952 | M   | 1            | 19 1/2       | 4 1/2              |                                  | +                     | +                          |
| Asch, D. P., and R. M.                          | [17] | 1953 | F   | 1            | 2            | 8 1/2              |                                  | +                     | +                          |
| Asch, D. P., and R. M.                          | [18] | 1954 | M   | 1            | 5            | 10                 |                                  | +                     | +                          |
| Asch, D. P., and R. M.                          | [19] | 1954 | M   | 1            | 10           | 12 1/2             |                                  | +                     | +                          |
| Asch, D. P., and R. M.                          | [20] | 1955 | M   | 1            | 8            | 12 1/2             |                                  | +                     | +                          |
| Asch, D. P., and R. M.                          | [21] | 1955 | M   | 1            | 4            | 13                 |                                  | +                     | +                          |
| Asch, D. P., and R. M.                          | [22] | 1955 | M   | 1            | 12 1/2       | 5                  |                                  | +                     | +                          |
| Asch, D. P., and R. M.                          | [23] | 1957 | M   | 1            | 3            | 10                 |                                  | +                     | +                          |
| Asch, D. P., and R. M.                          | [24] | 1957 | M   | 1            | 4            | 10 (1)             |                                  | +                     | +                          |
| Asch, D. P., and R. M.                          | [25] | 1957 | M   | 1            | 19 1/2       | 4 1/2              |                                  | +                     | +                          |
| Asch, D. P., and R. M.                          | [26] | 1958 | M   | 1            | 5            | 10                 |                                  | +                     | +                          |
| Asch, D. P., and R. M.                          | [27] | 1958 | M   | 1            | 11           | 21 (1)             |                                  | +                     | +                          |
| Asch, D. P., and R. M.                          | [28] | 1958 | M   | 1            | 6            | 18                 |                                  | +                     | +                          |
| Asch, D. P., and R. M.                          | [29] | 1959 | M   | 1            | 37           | 4                  |                                  | +                     | +                          |
| Asch, D. P., and R. M.                          | [30] | 1959 | M   | 1            | 1 1/2        | 0 (1)              |                                  | +                     | +                          |

in whom neither hyperoxaluria nor extrarenal deposits of calcium oxalate have been demonstrated. Patients in this category have been described in age ranging from 5 months to 2 years [72-76]. (2) Patients with calcium oxalate nephrolithiasis or nephrocalcinosis or both in whom extrarenal deposits of calcium oxalate have been demonstrated at autopsy but in whom the disorder had its onset in middle adult life or occurred concomitantly with and perhaps as a consequence of another serious renal disease [16, 77-78]. (3) Patients with calcium oxalate nephrolithiasis and hyperoxaluria but without nephrocalcinosis or known tissue deposits of oxalate in whom symptomatic disease first began in adult life [50-79].

A number of patients of the first two groups have been excluded from Table 14.1 because of incomplete pathologic studies of tissues or because of onset of the disease in adult life. Further study may disclose that the adult cases are indeed authentic examples of the primary disorder, but until more is known about conditions leading to secondary hyperoxaluria they had best be placed in an uncommitted category. Patients of the third group are discussed more fully below in connection with the general problem of oxalate nephrolithiasis.

## OXALATE NEPHROLITHIASIS

### INCIDENCE AND ETIOLOGIC FACTORS

In the United States from 23 to 70 per cent of all urinary tract stones are composed of oxalate [80-86]. This wide discrepancy reflects different criteria for classification. In the so-called "stone belt" of central Europe the frequency of oxalate stones is estimated as high as 65 per cent of all urinary tract calculi [87].

The etiology of oxalate nephrolithiasis is poorly understood. Oxalate calculi may form in acid, neutral, or alkaline urine and remain insoluble in either acid or alkaline urine. Neither infection nor stasis is so important a predisposing factor in oxalate nephrolithiasis as in calcium phosphate nephrolithiasis [1-31, 86].

In a number of areas of the world where oxalate stones are particularly prevalent, a high oxalic acid content of the diet has been implicated as an etiologic factor. In other areas where oxalate stones are common the diet is extremely low in oxalic acid content [31, 87-89].

Normal urine is usually supersaturated with calcium oxalate up to 14 or eightfold according to Joly [27-31]. The increased solubility of calcium oxalate in urine over that in water has been attributed to the presence of urea and colloidal substances [29, 90]. It has been suggested that oxalate nephrolithiasis results from decreased solubility of calcium oxalate in urine because of a disturbance involving such a protective colloidal mechanism but this remains to be demonstrated. At times hypercalcaemia is a contributing factor [50]. In most patients it is

found a prompt and considerable incorporation of the isotope into urinary oxalate. When constancy of isotopic enrichment had been achieved urinary oxalate was 50 and 32 per cent as highly labeled as free urinary glycine in the 2 subjects in whom glycine enrichment was measured. Thus approximately the same fraction of urinary oxalate appeared to be derived from glycine in these 2 hyperoxaluric subjects as in 1 control subject.

In one hyperoxaluric subject, Wyngarden, Verner and Elder [63, 79] found incorporation of 0.22 per cent of a single oral tracer dose of glycine  $1\text{-C}^{14}$  into urinary oxalate. This value is some fourfold greater than the mean normal value. This patient excreted 103 to 143 mg oxalic acid dihydrate per day, an amount about fivefold greater than the mean normal excretion value. Thus these results also suggest that about the same percentage of urinary oxalate is derived from glycine in this subject as in normal subjects.

Even in the most marked cases of hyperoxaluria the urinary excretion of oxalate amounts only to approximately 0.5 per cent of the total daily glycine turnover [64]. No studies have been performed with labeled glyoxylate or ascorbic acid in hyperoxaluric subjects.

## THE METABOLIC DEFECT IN PRIMARY HYPEROXALURIA

The data presented above indicate that the large amount of oxalate present in the urine and tissues of patients with oxalosis is of endogenous origin. They show that glycine is a precursor of about the same percentage of the urinary oxalate in patients with primary hyperoxaluria as in control subjects. The basic abnormality in primary hyperoxaluria is therefore probably a failure of one or another of the reactions by which glyoxylate is metabolized other than to oxalate, viz. transamination to glycine, reduction of glycolic acid or oxidative degradation to formate and carbon dioxide rather than excessive endogenous production of glyoxylate from glycine.

Whatever the defect it appears to be severe and lifelong. No cofactors are known that are unique to glyoxylate metabolism and no metabolic defect is known to exist in hyperoxaluric subjects other than overproduction of oxalate. It is reasonable therefore to postulate that an enzymatic step concerned directly or indirectly with glyoxylate metabolism may be defective in this disorder.

## RELATED CASES OF UNCERTAIN CLASSIFICATION

There are three groups of patients in whom certain features suggest the diagnosis of primary hyperoxaluria and oxalosis but in whom the actual relationship if any to the authentic syndrome is at present unclear. These are (1) Patients with nephrocalcinosis due to calcium oxalate

[63] Prior to dietary protein restriction the miscible pool of oxalate was 39.1 mg and its turnover 110 mg per day following decline of oxalate excretion the miscible pool was 1.8 mg and its turnover 8.3 mg per day. Thus the decline in excretion was secondary to a remarkable decrease in the content of soluble oxalate within body fluids. The great increase in turnover (and excretion) demonstrated on initial studies could have been secondary to increased production or increased absorption of oxalate. Subsequently an oxalate absorption study was conducted in the fasting state at a time when urinary oxalate was low [64]. Forty-four per cent of a 50 µg dose of oxalic acid  $1-2\text{ C}^{14}$  was absorbed and appeared in urine. This was somewhat greater than was found under similar conditions in 11 control subjects (one hypercalcemic) who excreted 24 and 35 per cent of the oral dose as urinary oxalate.

#### EXPERIMENTAL OXALATE NEPHROLITHIASIS OXALATE NEPHROCALCINOSIS AND HYPEROXALURIA

Keyser [94-95] produced oxalate calculi in dogs and rabbits following subcutaneous administration of oxalic acid esters and calcium salts. Rost produced renal calculi in dogs by feeding sodium oxalate [94a]. Hammarsten [95-96] produced oxalate stones in rats on a magnesium-poor diet. A deficiency in vitamins A and D as well as additional oxalates in the diet favored stone formation but was not essential since oxalate stones also formed in magnesium-deficient rats on an oxalate-free diet. The magnesium deficiency per se was not specific for oxalate calculus formation. These stones predominated when urine pH was acid whereas calcium phosphate stones formed when it was alkaline.

Gershoff and associates [97] produced an experimental vitamin B<sub>6</sub> deficiency in the cat characterized by failure of growth, emaciation, convulsions, anemia, and oxalate nephrocalcinosis. Excessive endogenous formation of oxalate led to hyperoxaluria but gross calculi were not seen at any level of the urinary tract. Extrarenal deposits of calcium oxalate were not detected but the experiments were of relatively short duration. Extensive glomerular lesions and deep cortical scars unlike the renal lesions of human oxalosis were observed in these cats. By feeding oxalic acid and oxamide, Fbstein and Nicolauer [98] produced renal lesions in dogs which were comparable to those formed in the vitamin B<sub>6</sub>-deficient cats.

#### GENETICS

Archer and associates [99] have made detailed studies of urinary oxalate excretion among relatives of three patients proved to have primary hyperoxaluria. No abnormally high level of urinary oxalate was encountered among the siblings, parents, first cousins, uncles, and aunts and more distant relatives. Among deceased relatives a history of

probable that transient dehydration and formation of very concentrated urine are important predisposing factors in oxalate stone formation

### OXALATE EXCRETION

Urinary oxalate excretion is normal in most patients who form oxalate stones [50, 91, 92], factors other than hyperoxaluria appear to be responsible. Nevertheless as pointed out above there exists a group of patients who form calcium oxalate stones with varying frequency, in whom hyperoxaluria of mild or even severe degree has been found. The relationship to the syndrome of primary hyperoxaluria is obscure.

In 1957, Dempsey [50] reported studies of oxalate and calcium excretion in a group of 45 patients with calcium oxalate stones in selected patients with idiopathic or secondary hypercalcaemia and in patients with hepatic cirrhosis. Ten subjects had hyperoxaluria (50 to 110 mg per day) including 5 of 11 patients with hepatic cirrhosis (70 to 80 mg per day). In this study all the hyperoxaluric subjects who formed calcium oxalate stones also had hypercalcaemia but a number of the hypercalcaemic patients had normal values for urinary oxalate. In one young patient who had primary hyperoxaluria and who was subsequently shown by Godwin et al. [17] to have oxalosis urinary oxalate excretion ranged from 130 to 180 mg per day and the urinary calcium excretion was normal. In other patients with primary hyperoxaluria.

A study [54, 79] of 12 selected patients with a long history of recurrent nephrolithiasis due to calcium oxalate stones may also be cited. In this group 4 patients excreted normal quantities of oxalate in urine [12 to 45 mg per day as  $(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$ ], 3 excreted between 50 and 100 mg per day and 5 excreted from 103 to 238 mg per day. In 2 patients of the latter group values of urinary oxalate eventually declined to normal. In 1 in whom no dietary changes were made values declined from 110 to 41 mg per day during approximately 4 months of observation. In the other (who also showed hypercalcaemia) dietary restriction of oxalate protein and calcium intake was accompanied by a prompt decline in urinary oxalate from 117 to 11 mg per day. Concomitantly this patient's urine became free of crystals of calcium oxalate for the first time in several years.

### METABOLIC STUDIES

Studies of incorporation of glycine  $1\text{-C}^{14}$  into urinary oxalate were performed in the adult hyperoxaluric hypercalcaemic subject [79]. The test was done during dietary restriction when urinary oxalate had declined to 54 mg per day. The patient incorporated 0.047 per cent a normal value. Additional studies are necessary to determine the origin of the urinary oxalate in this subject.

A limited number of additional observations was made on this patient

and biochemically by a continuous high urinary oxalate excretion. Extra renal deposits of calcium oxalate occur as a stage in the natural history of the disease. Their presence establishes the additional diagnosis of oxalosis. The disorder usually begins in early childhood and leads to death from renal failure in childhood or early adult life. No effective therapy is known.

2. The hyperoxaluria is due to a metabolic defect leading to endogenous overproduction of oxalate. About the same percentage of urinary oxalate arises from glycine in hyperoxaluric subjects as in normal subjects. On theoretical grounds a defect in metabolism of glyoxylic acid, the only known direct precursor of oxalic acid, is suspected but a precise biochemical localization of the metabolic defect is not yet possible.

3. The disorder is thought to be genetically determined and preliminary evidence suggests that it may be inherited as an autosomal recessive trait. No increase in urinary excretion of oxalate has been found in individuals who are presumed to be heterozygous for the trait.

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gestive of primary hyperoxaluria was obtained in only one sibling of one of the propositi. There was no history of consanguinity in any of the three families.

Archer et al. have made several cogent preliminary generalizations: (1) They point out that no relevant abnormality has been established in the parents of a single case of primary hyperoxaluria except for the father of Dunn's [9] case who had passed a urinary calculus. Urinary oxalate determinations were not recorded in Dunn's study. (2) No cases arising from consanguineous parents have yet been reported. (3) Males predominate but females with this disorder are afflicted with an equally severe disease. (4) Most of the cases have occurred as isolated examples in a family but both News and Black [5] and Aponte and Fetter [6] have reported affected siblings including identical twins in the latter family. Archer et al. suggest that these data are compatible with the hypothesis that primary hyperoxaluria is due to the operation of a rare recessive character.

Since the urinary excretion of oxalate was normal in all family members studied by Archer et al. [99] the detection of individuals heterozygous for the defective gene is presently impossible. Perhaps a suitable stress such as oral loading doses of glycine or glyoxylate would lead to hyperoxaluria and permit their detection. One wonders whether any of the adult subjects with recurrent oxalate stones and possibly transient hyperoxaluria may represent such carriers of the gene for primary hyperoxaluria responding to an unknown provocative stress with an increase in endogenous production of oxalate.

The possibility that genetic factors may influence stone formation without operating through mechanisms concerned with oxalate production cannot be excluded on the basis of available evidence. In 1931 Gram [100] published an extensive pedigree of five generations of a family in which a number of cases of calcium oxalate urolithiasis had arisen. Urinary oxalate determinations were not reported and the clinical courses did not suggest that they were examples of primary hyperoxaluria.

## TREATMENT IN PRIMARY HYPEROXALURIA

No successful treatment directed at the fundamental clinical problem persistent hyperoxaluria is available. Measures shown to reduce excretion of oxalate in certain other patients with hyperoxaluria viz. protein restriction and administration of vitamin B<sub>6</sub> and of sodium benzoate warrant further trial.

## SUMMARY

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## Chapter 15

### Maple Syrup Urine Disease\*

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*Joseph Dancis and Mortimer Ient*

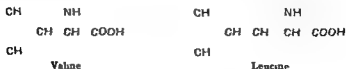
#### HISTORICAL NOTE

In 1954 Menkes Hurst and Craig described a family in which four out of six infants one girl and three boys died during the first weeks of life with what appeared to be a congenital metabolic disease [1]. The prominent signs were vomiting muscular hypertonicity and a maple syrup odor to the urine. Examination of the urine by paper chromatography failed to reveal an abnormality in the amino acid pattern and no clue could be found to the metabolic anomaly.

In 1957 Westall Dancis and Miller described a 20 month-old infant (Boy A) who was mentally retarded had muscular hypertonicity and a maple syrup odor to the urine [2]. A detailed account has appeared more recently [3]. An excess of leucine was found in the urine of this patient on analysis by paper chromatography. Further investigation (4) suggested a metabolic block in the degradative pathway of the branched chain amino acids. An additional finding was an elevated methionine and low cystine level in the plasma. Mackenzie and Woolf [5] have reported a second case (Girl B) with the same metabolic anomaly.

#### METABOLISM OF THE BRANCHED CHAIN AMINO ACIDS

Valine leucine and isoleucine are referred to as the branched chain

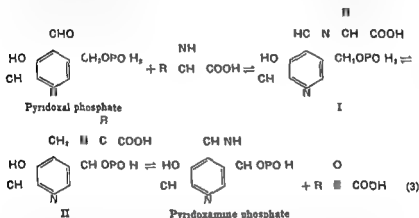


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specific [10] Even the more specific transaminases will occasionally retain activity when the receptor or donor is replaced by a substrate of similar structure [9]

The cofactor which mediates transamination is vitamin B<sub>6</sub> in either the pyridoxal or pyridoxamine form [11] A plausible mechanism which explains the coenzymatic nature of the vitamin is shown in Reaction 3 [11-12] A reversible enzymatic reaction between pyridoxal phosphate and the amino acid on the enzyme surface produces the Schiff base (I)

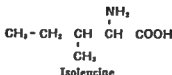


Simultaneous hydrogen and double bond shifts result in the isomeric Schiff base (II) which then splits into the keto acid and pyridoxamine phosphate. The reversibility of the reaction permits the transfer of the amino group from pyridoxamine phosphate to the keto acid acceptor which is usually  $\alpha$ -ketoglutarate. This completes the transamination reaction.

The  $\alpha$ -keto acids are degraded further by irreversible oxidative decarboxylation (Reaction 4)



This reaction has been well documented for pyruvic and  $\alpha$ -ketoglutaric acids. Direct evidence for the participation of the branched chain keto acids is lacking, probably because the decarboxylated acid is rapidly metabolized to products of greater interest to investigators. Kinnery, Takeda, and Greenberg [13] demonstrated the conversion of valine to  $\alpha$ -ketovaleric and isobutyric acid by rat liver homogenate. Presumably degradation occurs through the keto acid to isobutyric acid. Bloch [14] found that the rat degrades leucine and isovaleric acid to acetate in a similar fashion and suggested that leucine is converted to



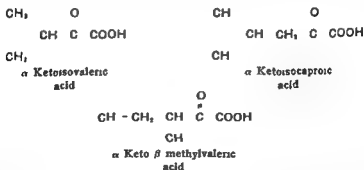
amino acids because each contains a methyl group which is not part of the longest carbon to carbon sequence. In common with the other amino acids, the branched chain amino acids are either used in protein or polypeptide synthesis or are degraded extensively.

The catabolism of valine, leucine and isoleucine is initiated by the loss of the amino group either by oxidative deamination (Reaction 1) or by transamination (Reaction 2). In oxidative deamination the enzyme L-amino acid oxidase liberates ammonia with the formation of the  $\alpha$  keto acid. The branched chain amino acids are relatively good substrates for



L-amino acid oxidase but the low enzymatic activity and the limited distribution of the enzyme make this pathway insignificant [6].

On the other hand transamination occurs in most animal tissues, and virtually all the natural amino acids participate [7]. Transamination differs from oxidative deamination in that free ammonia is not involved [8]. Alpha ketoglutaric acid is the most common of several  $\alpha$  keto acids which may function as an  $-\text{NH}_2$  receptor in enzymatic transamination. It couples effectively with valine, leucine and isoleucine converting them to the respective  $\alpha$  keto acids:  $\alpha$  ketoisovaleric acid,  $\alpha$  ketoisocaproic acid and  $\alpha$  keto  $\beta$  methylvaleric acid [9].

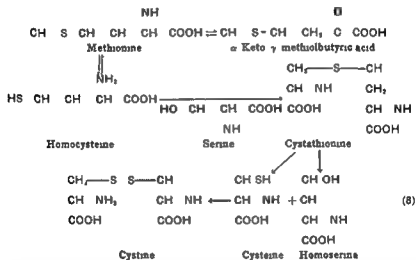


The literature on the specificity of the transaminases is too extensive and at times too contradictory to be reviewed here. Some transaminases appear to be effective against several amino acids while others are more

## METABOLISM OF METHIONINE

In contrast to the branched chain amino acids methionine has two major metabolic pathways available to it. Methionine transaminates to give  $\alpha$  keto- $\gamma$  methylbutyric acid [8] but to the authors knowledge the extent of further degradation is not known. Methionine is converted to other amino acids by enzymatic reactions involving sulfur.

The conversion of methionine to cystine was demonstrated unequivocally by isotope experiments [20-21]. Homocysteine [22], serine [23-24] and cystathionine [25] were shown to be intermediates. The sequence is as follows (Reaction 8).



Methionine is converted to homocysteine in an enzymatic reaction which utilizes adenosine triphosphate [26] to reduce the energy barrier to transmethylation. Several methyl acceptors are known. A common one is guanidoacetic acid which is eventually converted to creatinine. Homocysteine may be reconverted to methionine with betaine acting as the methyl donor. Homocysteine and serine condense to form cystathionine which in turn cleaves to cysteine and homoserine. It can be seen that serine is the sulfur acceptor. The condensation and cleavage require different enzymes both requiring pyridoxal phosphate as a co-factor. The oxidation of cysteine to cystine occurs spontaneously but the enzymatic conversion is also known [27]. Further metabolic pathways of cysteine and cystine are apparently of no importance in the disease.

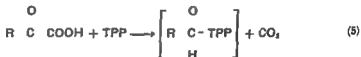


isovaleric acid probably through  $\alpha$  keto isopropionic acid Coon and Abrahamson [15] have presented similar indirect evidence that  $\alpha$  methyl butyrate is an intermediate in the metabolism of isoleucine and they postulate that  $\alpha$  keto  $\beta$  methylvalerate is an intermediate in the conversion

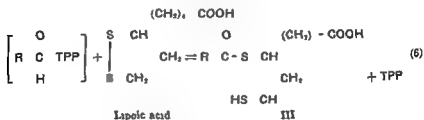
Leucine 1  $C^{14}$  is readily decarboxylated by rat brain liver, and kidney [4] Rabbit white blood cells obtained from peritoneal exudate are also effective but rabbit and human whole blood give negative results

The details of this complex enzymatic process have been deduced from studies with bacteria [16] but there is evidence that the pathway is similar in animal tissue [17] In the reactions to be described pyruvate and  $\alpha$  ketoglutarate were used as substrates Although preliminary unpublished work suggests that the branched chain  $\alpha$  keto acids behave similarly [18] definite conclusions are not warranted at this time

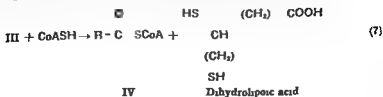
Several enzymes and cofactors are involved in the decarboxylation of  $\alpha$  keto acids [10-19] Thiamine pyrophosphate (TPP) in a reaction not clearly understood catalyzes the elimination of carbon dioxide and becomes bound to a fragment which is probably at the aldehyde oxidation level (Reaction 5)



Another essential cofactor lipoic acid which is tightly bound to the enzyme oxidatively displaces TPP (Reaction 6) A bond forms between dihydrolipoic acid and the decarboxylated acid (III)



Coenzyme A (CoASH) displaces dihydrolipoic acid to produce an acyl CoA compound (IV) (Reaction 7) Diphosphopyridine nucleotide (DPN) and flavin adenine dinucleotide (FAD) are two more cofactors Further degradative pathways are reviewed elsewhere [28]



(Reactions 5 to 7) The first two acids were sought in the urine with gas phase chromatography. None of the urinary peaks coincided with isovaleric acid (Table 15.1) but one did correspond with isobutyric acid. However, the area underlying the peak was considerably less than that of the keto acids. The reverse would be expected if the metabolic block were beyond isobutyric acid. The metabolic block is therefore presumed to exist between the keto acids and the simple acids and at the point of oxidative decarboxylation of the keto acids.

TABLE 15.1 GAS PHASE CHROMATOGRAPHY OF ORGANIC ACIDS IN PATIENT'S URINE

| Peak | Retention time | Compound   | Percentage of total |
|------|----------------|--|---------------------|
| 1    | 5              |  | 4.1                 |
| 2    | 5.6            | $\alpha$ Ketoisovaleric acid ( ketovaline )                        | 1                   |
| 3    | 7.8            | $\alpha$ Keto $\beta$ -methylvaleric acid ( ketoisoleucine )       | 55                  |
| 4    | 8.7            | $\alpha$ Ketoisocaproic acid ( ketoleucine )                       | 8                   |
|      |                | $\alpha$ Hydroxyisovaleric acid ( hydroxyvaline )                  |                     |
| 5    | 13.1           | $\alpha$ Hydroxy $\beta$ -methylvaleric acid ( hydroxyisoleucine ) | 5                   |
|      |                | $\alpha$ Hydroxyisocaproic acid ( hydroxyisoleucine )              |                     |
| 6    | 22.5           |  | 0.4                 |
| 7    | 26.9           |  | 0.2                 |

The acids were converted to the methyl esters and packed in the gas phase over polydiethylene glycol succinate.

An unsuccessful attempt was made to demonstrate the postulated aldehyde intermediate (Reaction 5). If it were present in excess and in a form capable of reacting with 2,4-dinitrophenylhydrazine, it should have the solubility characteristics of a neutral hydrazone. No such compound could be demonstrated in the urine. This observation is obviously open to more than one interpretation.

Mackenzie and Woolf [5] have reported an excess of the branched chain amino acids and keto acids in the urine of Girl B. It seems most probable that there is a similar metabolic block in their patient.

The specific involvement of the three branched chain keto acids indicates that there is a common step in their metabolic pathways and suggests that an enzyme associated with their further degradation is deficient. It is well to keep in mind that alternative theories can be constructed to fit the currently available data. This aspect and the need for further study are discussed below in the appropriate section.

## OTHER METABOLITES

The organic acids of the urine of Boy A were extracted and converted to the methyl esters for analysis by gas phase chromatography. Seven

## THE 'METABOLIC ERROR'

In the investigation of Boy 1 by Westall et al. amino acid analysis of the urine by column chromatography revealed increased excretion of leucine, isoleucine, and valine. There was also roughly a tenfold increase in the plasma concentrations of the branched chain amino acids (Fig. 15-1). This indicated that the aminoaciduria was of the overflow type.

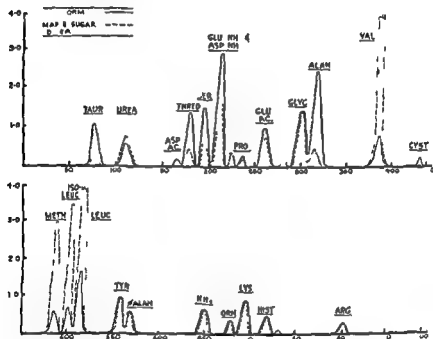
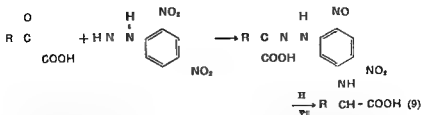


Fig. 15-1 Plasma amino acid pattern determined by column chromatography. The patient's levels (broken line) are compared with those of a normal subject (continuous line) [9].

and suggested a metabolic block in the degradative pathway of the amino acids. Other abnormalities were noted in the plasma amino acid pattern, particularly a high methionine and low cystine level. However, the elevations in the branched chain amino acids were more striking and therefore this aspect has received primary attention.

Tissues obtained from Boy 1 at autopsy were able to transfer the amino group from the branched chain amino acids to  $\alpha$ -ketoglutarate (Reaction 2), indicating that a block lay at a lower level. This was confirmed when an excess of  $\alpha$ -keto acids was found in the urine by paper chromatography [29] and by gas phase chromatography [4].

The next metabolic step is normally that of oxidative decarboxylation of the keto acids to isobutyric, isovaleric, and  $\alpha$ -methylbutyric acids.



The keto acids can also be identified by paper chromatography of the 2,4-dinitrophenylhydrazones derivatives [30] and by gas phase chromatography of the methyl ester derivatives. For the last two methods pure samples of the keto acids are needed as standards. These are difficult to obtain commercially but can be synthesized enzymatically [31].

The demonstration that the simple branched acids isobutyric, isovaleric and  $\alpha$ -methylbutyric acids are not present in excess will complete the diagnostic work up and localize the defect as accurately as is possible at present.

## METHODS

### *Qualitative Demonstration of Excess Keto Acids*

- 1 To 1 ml urine add 4 ml 2,4-dinitrophenylhydrazine in 2N hydrochloric acid. A yellow precipitate forms.
- 2 After 10 min extract the precipitate with ether.
- 3 Extract the ether with 10 per cent sodium carbonate. In a positive test the yellow color enters the water phase.

### *Identification of the Keto Acids [30]*

- 1 Begin as above but use a larger aliquot of urine (about 20 ml).
- 2 Acidify the sodium carbonate extract and extract with peroxide-free ether.
- 3 Distill off the ether and replace with 30 ml water.
- 4 Hydrogenate the mixture until it is colorless. This can be done in a hydrogenator over 75 mg activated platinum. An electrolytic desalter will accomplish the same result.
- 5 Identify the reconstituted amino acids by paper chromatography.

### *Quantitative Analysis for Keto Acids [32]*

- 1 To 1 ml urine add 8 ml 15 per cent trichloroacetic acid and 500 mg Lloyd's reagent Zeolite or Irmumat. Shake and centrifuge.
  - 2 To a 1 ml aliquot of supernatant add 0.2 ml 2,4-dinitrophenylhydrazine solution and mix. Let stand 15 min.
  - 3 Add 1.5 ml *m*-xylene. Mix vigorously over 2 min period. Centrifuge.
  - 4 To a 1 ml aliquot of the supernatant add 1.5 ml 10 per cent NaCO. Mix vigorously and centrifuge. Decant the upper phase.
  - 5 To 1 ml of lower layer add 1 ml of 1.5N NaOH. After 10 min read at 570 m $\mu$ .
- For blank use 1 ml water instead of urine.

peaks were demonstrated. Three had the same retention times as standard samples of the branched chain keto acids. A fourth peak coincided with two of the  $\alpha$  hydroxyacid derivatives of the branched chain amino acid. The third hydroxyacid overlays one of the keto acids (Table 15.1). Three of the peaks were not identified. Thus there is presumptive evidence for the presence of the hydroxyacid. Mackenzie and Woolf have stated that there were "non keto acids with the properties of  $\alpha$  hydroxy acids" in the urine of Girl B but no further details are given in their report.

*Indoleacetic* and *indolelactic acids* have been identified by paper chromatography in the urine of Boy A and Girl B and appear to be present in excess amounts.

The compound or compounds responsible for the maple syrup odor is not known.

### DIAGNOSIS

A clinical diagnosis is suggested by an infant presenting with central nervous system symptoms (vomiting, poor feeding, muscular hyper-tonicity) beginning possibly a week after birth and with a maple syrup odor to the urine. Mental retardation rapidly becomes evident. Variants of this clinical picture may be recognized with further experience. If Menkes' original patients suffered from the same metabolic anomaly as Boy A and Girl B then the disease varies from the acutely lethal to one compatible with existence for at least 2 years. A second infant with this disease has been born to the family of Boy A, confirming the familial nature of the disease. The family of Boy A gave a definite history of waxing and waning of the maple syrup odor; at times the odor could not be detected at all. The diagnosis must therefore be suspected even in the absence of odor. In this preliminary state of knowledge it would be wise to test for ketoaciduria (see below) in all cases of suspected congenital metabolic disease.

*Amino acid analysis* of the urine or plasma may reveal an excess of the branched chain amino acids. The plasma methionine may also be elevated. The urinary levels of the branched chain amino acids in Boy A were normal at 10 months of age even though the branched chain keto acids were significantly elevated.

A *ketoaciduria* may be demonstrated quantitatively or qualitatively. The 24 hr excretion in Boy A was 49 mg and 252 mg at 6 months and 18 months of age respectively. This contrasts with figures from three normal infants of 4, 2, and 3 mg per 24 hr.

The demonstration of ketoaciduria is useful as a screening test. In order to establish further the diagnosis the keto acids must be identified as derivatives of the branched chain amino acids. This is best done by converting the keto acid to the hydrazone which is reduced to the parent amino acid and then identified by paper chromatography (Reaction 9).

in the conversion of methionine to cystine. There were also considerably reduced levels of other amino acids (Fig 15-1). The relation to the primary metabolic defect is not clear.

The compound responsible for the maple syrup odor has not been identified. The keto acids have no such odor but it may be due to a derivative or decomposition product. In the case of Boy A, a reliable history was obtained that the maple syrup odor was not always present. The authors have noted a similar odor in impure samples of fumaric acid, pyruvic acid, citric acid, and  $\alpha$  hydroxybutyric acid. It is possible that a maple syrup odor may be caused by more than one compound or group of compounds and it is also possible that maple syrup urine diseases may be associated with other metabolic anomalies. A recent report provides suggestive support for the hypothesis [83]. The details of this case are not yet available but there was an increased excretion of the branched chain amino acids and phenylpyruvic acid and a burnt sugar odor to the urine.

#### GENETICS

The disease appears to be familial but its pattern is not yet clear. Boy A was one of three children. One sibling is normal and a second has just been born with the disease. The parents are unrelated and no history can be obtained of a similar disease on either side. Girl B is an only child and no significant genetic history is reported.

#### THERAPY

The possibility of treatment by a diet low in the branched chain amino acids is suggested by the success of low phenylalanine diets in phenylketonuria (Chap 10). Essential to the success of such an approach is that the infant be normal or at least not irreversibly damaged at birth. There is so far no information on this aspect of maple syrup urine disease.

#### POSSIBILITY OF OTHER KETOACIDURIAS

Transamination is generally the first metabolic step in degradation of the amino acids and oxidative decarboxylation is commonly the second. It would be surprising indeed if other ketoacidurias are not recognized eventually.

#### SUMMARY

Two unrelated infants have been reported with metabolic diseases characterized by early onset of feeding problems, retarded development, and a maple syrup odor to the urine. In both instances the keto acids of leucine, isoleucine, and valine were found in the urine, suggesting a block at the step of oxidative decarboxylation. In one infant the plasma amino acid were investigated. In addition to an elevation of the branched chain

*Reagents*

1. 2,4-Dinitrophenylhydrazine solution Grind 100 mg of the compound. Add 8 ml water mix and follow with 15 ml concentrated HCl. Shake in shaking machine for 30 min. Allow to settle and use supernatant.

■ Standard Prepare 0.01 M standard solution in 0.1 N H<sub>2</sub>SO<sub>4</sub>. The branched chain keto acids are difficult to obtain commercially. Pyruvic acid can be used. Commercial samples frequently must be recrystallized. The difference in extinction coefficients among these keto acids does not introduce a clinically significant error.

## ARFAS FOR FUTURE STUDY

## CLINICAL COURSE

At present there have been three reports of cases with a maple syrup odor to the urine associated with early signs of central nervous system disease. More observations are needed to outline the natural history of the disease.

## BIOCHEMISTRY

The primary metabolic anomaly can not yet be considered established. The accumulation of the branched chain keto acids suggests a block at the next enzymatic step—that of oxidative decarboxylation. However, similar findings could result from overloading a normal enzyme of limited capacity. The high methionine and low cystine levels suggest another metabolic block. This aspect requires further investigation. It is possible that the primary anomaly lies in the metabolism either of methionine or of the branched chain amino acids or it may be that both anomalies have another as yet unidentified origin.

A biochemical interpretation of the symptoms is lacking. One may suspect that the symptoms arise from an excess of metabolites proximal to the block or derivatives of these. No important metabolic function has been attributed to the compounds distal to the block.

The preliminary identification of indole acids in the urine of maple syrup urine disease deserves further investigation. In phenylketonuria it has been demonstrated that the indolaciduria is associated with decreased formation of serotonin (5-hydroxytryptamine) and it has been suggested that this may be the primary factor in the mental deficiency. Certainly similar studies should be done in maple syrup urine disease when possible. It would be wise to keep in mind that the branched chain keto acids and hydroxyacids resemble certain essential normal metabolites and that their accumulation in excess might interfere with normal metabolism in more than one area.

Other amino acid abnormalities in addition to the accumulation of the branched chain amino acid were noted in the plasma of Boy A. The most striking was an elevated concentration of methionine (3 mg per 100 ml) and a level of cystine too low to be detected. This suggests a block

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amino acids the plasma methionine was high and the cystine low, suggesting that a block may also exist in this pathway. Further study is needed to define the metabolic anomaly better.

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#### **Part Four**

### **Diseases Characterized by Evidence of Abnormal Lipid Metabolism (The Lipidoses)**

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## Chapter 16

### Essential Familial Hyperlipidemia

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Donald S. Fredrickson

The term *lipidosis* has been used to describe almost any disturbance of lipid metabolism, particularly one leading to abnormal accumulations of lipid in blood or in certain organs. A *primary familial lipidosis* by definition should arise from a genetically transmitted abnormality in lipid metabolism. Five currently recognized diseases appear to meet this definition. These may be conveniently subdivided into two groups:

- 1 Primary familial lipidoses characterized by hyperlipidemia (the essential hyperlipidemias)
  - a Essential familial hyperlipemia
  - b Essential familial hypercholesterolemia
- 2 Primary familial lipidoses characterized by intracellular accumulation of sphingolipids (the sphingolipidoses)
  - a Sphingomyelinosis (Niemann Pick disease)
  - b Gangliosidosis (infantile amniotic family idiocy)
  - c Cerebrosidosis (Gaucher's disease)

Each of these conditions represents a more or less distinct clinical syndrome that usually can be accurately diagnosed. A well understood biochemical lesion has not yet been demonstrated for any of them, however, and it is possible that several specific diseases, not all necessarily familial, will eventually be separated from within each syndrome. It is also possible that the inheritable abnormality in some of them may yet prove to involve the metabolism of protein or carbohydrate rather than lipid.

A number of lipidoses have obviously been excluded from the above classification. Many of these are lipidoses secondary to other diseases such as xanthomatous biliary cirrhosis, the nephrotic syndrome, or diabetes. Others such as the hyperlipidemia seen in glycogen storage disease may be secondary to other inheritable molecular diseases. The



when more specific diagnosis cannot be made.<sup>1</sup> When appropriate the type of xanthoma present may be added as a subordinate diagnosis.

## BLOOD LIPIDS AND LIPOPROTEINS

Adequate description of hyperlipidemia depends upon the definition of the normal state of lipids and lipoproteins in blood and extracellular fluid.

### BLOOD LIPIDS

In plasma from a normal human being there are usually 450 to 750 mg per 100 ml lipids (Table 16-1). Proper examination of the lipid content should be done on a specimen collected after an overnight (12 to 16 hr) fast. Either plasma or serum may be used. Either should be perfectly

TABLE 16-1 NORMAL SERUM LIPID CONCENTRATIONS IN THE FASTING HUMAN BEING

| Lipid   | Concentration<br>mg/100 ml |
|---|----------------------------|
| Cholesterol (10-75% esterified)   | 150-240                    |
| Phospholipids: approximate composition—lecithin (60%) sphingomyelin (10%) lysolecithin cephalin,inositol phosphatide others (10%) | 150-250                    |
| Glycerides  | 50-150                     |
| Cerebrosides  | 3-6                        |
| Carotenoids total   | 0.1-0.2                    |
| Unesterified fatty acids  | 0.3-0.6 (mEq/l)            |
| Total fatty acids: cholesterol ester (60%) phospholipid (20%) glyceride (25%) unesterified (5%)                                   | 200-400                    |

clear to transmitted light if the patient has properly fasted. If even faint lactescence (hyperlipemia) is visible, significant elevation in glyceride concentration is almost certainly present. Some hyperglyceridemia may be present, however, in perfectly clear serum.

### Chemical Analysis

By far the most widely determined blood lipid is cholesterol, for which a number of useful tests have been developed. The standard of reference for all these is still the reliable Schoenheimer-Sperry method [4].

Unless obvious hepatic disease is present, there is little to be gained, however, by separate measurement of free and esterified cholesterol in studying hyperlipidemia. Determination of total phospholipids and

<sup>1</sup>In the *Standard Nomenclature of Diseases* sponsored by the American Medical Association [1] only *familial hypercholesterolemia* is listed. The 10th edition of the *International Statistical Classification of Diseases* sponsored by the World Health Organization lists only the unsatisfactory terms *xanthomatosis* and *lipodystrophy* for classification of these syndromes [5].

"cholesterol lipidoses," represented by eosinophilic granuloma or the Hand-Schüller-Christian syndrome is nonfamilial and cannot be included either.

## DEFINITIONS

It is with misgivings that discussion of a subject already burdened with confusing terminology is begun with the introduction of another new term. *Essential hyperlipidemia* seems an appropriate generic term however for the group of lipidoses whose distinguishing feature is the presence of abnormally high concentrations of lipid in the extracellular fluid.

Sound argument could be advanced for the term *Essential hyperlipoproteinemia* since concentrations of some lipoproteins can be elevated without recognizable hyperlipidemia [1] but too few physicians have the facilities to measure lipoproteins, and lipid analyses alone are usually adequate for the purposes of diagnosis.

Unfortunately the perfectly good term *hyperlipemia* has too long been used in a restricted sense to permit return to its broader meaning. In keeping with tradition it must be used to define lactescence of plasma due to hyperglyceridemia. *Essential* will be used here in place of *primary* or *idiopathic*. The weight of historical tradition demands also that the term *essential hypercholesterolemia* be retained to define a more or less distinct clinical syndrome even though in this condition the phospholipid concentrations are almost always significantly elevated and often the glyceride levels are as well.

Much confusion can be avoided if the distinction between hyperlipidemia and xanthomatosis is maintained. Not all patients with essential hyperlipidemia develop xanthomas and the latter are most probably always a secondary manifestation dependent upon the hyperlipidemia. To emphasize the primary importance of the blood lipid changes the term xanthomatosis should be subordinated.

It cannot be assumed that all cases of essential hyperlipidemia spring from an inheritable disorder of lipid metabolism. The proper concern here is only with those which appear to be genetically transmitted. These may be considered in terms of two clinical syndromes: *essential familial hyperlipemia* (synonyms: idiopathic familial hyperlipemia, hépatosplénomégale lipoidose—type: Bürger (Rütz)) and *essential familial hypercholesterolemia* (synonyms: familial hypercholesterolemic xanthomatosis, primary hypercholesterolemic xanthomatosis, primary cholesterol lipidoses).

Since there are confusing cases of hyperlipidemia which have the features of both essential hypercholesterolemia and essential hyperlipemia (see below) it is suggested that until classification and diagnostic methods are improved the term *essential (familial) hyperlipidemia* be used.

## Phospholipids

The phospholipids and other complex lipids present more formidable chemical problems and exact analytical data for blood and tissue composition of many of the complex lipids are only slowly becoming available. Although it has been long known that most of the plasma phospholipids contained choline [19], recent use of silicic acid chromatography [20] has helped greatly in clarifying their composition. Lecithin (phosphatidyl choline) and sphingomyelin comprise almost 90 per cent of the plasma phospholipids. The remaining fraction has been reported to contain small

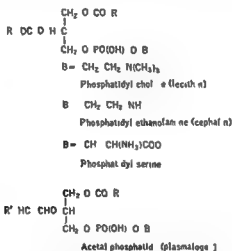


Fig. 16-1. Some naturally occurring phospholipids. There is recent evidence [110] that R is not R' [19] and represents the saturated acyl group on lecithin.

amounts of phosphatidyl ethanolamine, lysolecithin, phosphatidyl serine, inositol phosphatide, and possibly other minor amino-nitrogen-containing components. A small fraction of the phospholipids contain fatty acids as acetals.

In addition to the phosphorus-containing complex lipid, there are small quantities [21, 21a] of cerebroside, sugar-containing sphingolipids.

It is believed that all naturally occurring lecithins (Fig. 16-1) are of the *L-α* configuration. The same is probably true for phosphatidyl ethanolamine and other derivatives [22]. The specific acyl groups in each of the glycerophosphatides and their variation under different metabolic conditions remain to be established. Hanahan [22] has reported that the fatty acids in liver phospholipids are asymmetric: the unsaturated acid predominating in the  $\alpha$  position and saturated acids in the  $\beta$  position. In plasma, however, more than half the phospholipid fatty acids may be unsaturated.



glycerides requires no more complicated laboratory equipment but does demand technical skill especially in preparing quantitative lipid extracts which are free of nonlipid contamination. In such an extract total lipids are usually determined gravimetrically or by reduction of an oxidant such as potassium dichromate [5]. Total fatty acids may also be determined after saponification by titration or by weight or total carboxylic esters may be measured colorimetrically [6]. Total phospholipids are usually determined by phosphorus analyses. The values are expressed as phospholipid by using a commonly accepted mean value for phosphorus as 4 per cent of the total weight of the phospholipids [7].

Glycerides are most frequently determined as the residual lipid or fatty acids not specifically accounted for by phospholipid or cholesterol. For this reason normal values given for glyceride vary greatly in different laboratories. In the author's clinic, using the methods of Bragdon [5] which measure total oxidizable material in a chloroform-methanol-water extract of serum, one rarely sees glyceride values above 100 mg per 100 ml in a normal individual of any age. More specific chromatographic separations of plasma lipids are available [8] but are too time-consuming for routine use.

Of published methods for determining plasma unesterified fatty acid the method of Dole [9] is simplest but is still too complicated for wide clinical application.

Diurnal variations in plasma cholesterol and phospholipid levels may be ignored from the clinical standpoint. Glyceride concentrations may vary many fold; the highest concentrations occurring 3 to 5 hr after ingestion of fat. Unesterified fatty acid concentrations are subject to very wide variations in normal individuals. They are low after carbohydrate feeding and elevated during fasting, exercise or emotional stress [9, 10].

### Cholesterol

Cholesterol ( $\Delta^5$  cholestene- $3\beta$ -ol) is for all practical purposes the sole sterol in blood. Cholestanol (cholestane- $3\beta$ -ol) may be present in 1 to 3 per cent of the concentration of cholesterol [11]. Only 25 to 30 per cent of cholesterol molecules in plasma are the free alcohol. In the rest the single hydroxyl is joined through an ester linkage with a fatty acid. The acids are largely unsaturated [12] and in man may be over 80 per cent linoleic and oleic acids [13]. Except in clinically evident hepatic disease the ratio of esterified to total cholesterol is remarkably constant [14]. In certain of this will be explained later. Although the biologic functions of cholesterol are poorly understood, an enormous amount of information has been accumulated concerning its biochemistry. This information has recently been assembled in several texts [15, 16] and reviews [17, 18] to which the reader is referred for many details.

UFA circulate bound largely to albumin which contains sites capable of firmly binding several moles of UFA per mole of protein [31]. Small and possibly variable amounts of UFA also probably circulate bound to plasma lipoproteins [32] and possibly to erythrocytes [33-34]. The chemical composition of the plasma UFA is now being gradually defined [35-36] but has not yet been determined under a variety of conditions. As determined by vapor phase chromatography, the number of acids normally present in human plasma is large. It includes the saturated acids from  $C_{10}$  to  $C_{18}$  and significant amounts of long chain polyethenoid acids greater than  $C_{18}$  in length. Linoleic and oleic acids make up the largest components. Short term feeding experiments indicate that the plasma UFA are not greatly affected by the composition of the fat in the diet [39]. Their composition resembles the depot fats [37] but is somewhat higher in certain polyethenoids.

The evidence indicating the role of UFA in plasma transport to meet caloric demands has accrued from a number of experiments recently summarized elsewhere in detail [38]. The plasma concentration rises sometimes three to five-fold at times when requirements for oxidizable fat rise such as in fasting, uncontrolled diabetes, hyperthyroidism, or exercise. Supplying nonfat calories or restoring the capacity to oxidize glucose by giving insulin to diabetic patients quickly causes the plasma UFA concentration to fall [9-10]. It has been shown that UFA are avidly extracted by tissues when there is need to burn fat [39]. Studies with labeled fatty acids in man indicate that despite the tight binding to albumin, the UFA leave the plasma at turnover rates varying from 0.1 to 2 mEq per min [40-41].

The bulk of plasma UFA probably arises from the adipose tissues and is oxidized in liver, heart, skeletal muscles, and most other tissues with the possible exception of the brain. All available evidence indicates that utilization of UFA is mainly governed by controlling their release from the adipose tissue depots [38]. The mechanism of release has not yet been fully elucidated. It appears to be related intimately to some critical amount of carbohydrate utilization and possibly to certain humoral and neurogenic factors. Epinephrine, norepinephrine [42] and ACTH [43] have been demonstrated to cause UFA release from isolated adipose tissue. Most of the effects also cause the plasma UFA level to rise in the intact man or animal. Growth hormone also elevates plasma UFA concentrations [44]. Cholinergic blockade may prevent the UFA rise after physical stress. It is possible that the neurogenic stimulus is more primitive and separate from carbohydrate utilization as a regulator.

### Carotenoids

Carotenoids are aliphatic or alicyclic pigment usually containing 40 carbon atoms (8 isoprene unit). Both hydrocarbon (carotene) and

Lipsky et al [13] found 63 per cent linoleic and oleic acids in the plasma phospholipids in man

In lysolecithin one of the hydroxyl groups of glycerol is not esterified. The significance of these compounds in plasma is not known but it is of interest that this phospholipid has been reported to comprise the bulk of a small amount of lipid that is associated with protein in molecules of greater density than all other plasma lipoproteins [23] (see below)

The acetal phospholipids called *plasmalogens* by their discoverers Feulgen and Bersin [24] are only a very minor constituent of plasma phospholipids. The structure for them shown in Fig. 16-1 is that proposed by Rapport et al [25] and Debuch [26]. The  $\alpha\beta$  unsaturated ether linkage joining the fatty aldehyde to glycerol occurs predominantly at the  $\beta$  position in ox heart plasmalogens [27] but may depend upon the species from which the tissue is obtained [28]. Plasma acetal levels have been studied under different conditions but no specific relationship of acetal to hyperlipidemia has yet been reported. Since they account for 40 per cent or more of the phospholipid in the heart [27] and possibly in other tissues they may ultimately prove to have considerable physiologic importance.

The sphingolipids have a singular importance in the lipidosis characterized by intracellular accumulation of lipid. They are discussed in detail in Chaps. 17 to 19.

### Glycerides

Triglycerides are of great utility to the body, comprising the major storage form of fatty acids in the adipose tissue and the principal form in which exogenous fatty acids are transported from the intestine to the plasma. They may also be important in the net transport of endogenous fatty acids in the plasma but this has not been rigidly demonstrated experimentally. The relatively simple structure of the triglycerides belies the work yet to be done on fixing the nature and arrangement of the fatty acid moieties found in glycerides in various tissues and in the blood under a variety of circumstances.

Although the bulk of plasma glycerides are triglycerides [8] small quantities of di- and monoglycerides have been reported during both fasting and fat absorption [29-30]. The general term *glyceride* may be more proper in discussing plasma lipids whenever distinction between higher and lower forms is not made.

### UFA

The plasma unesterified (or nonesterified) fatty acids (UFA or NEFA) are almost negligible in concentration (Table 16-1) but may be more important than all the other lipids combined as the transport form of fat readily available for oxidation.

UFA circulate bound largely to albumin which contains sites capable of firmly binding several moles of UFA per mole of protein [31]. Small and possibly variable amounts of UFA also probably circulate bound to plasma lipoproteins [32] and possibly to erythrocytes [33-34]. The chemical composition of the plasma UFA is now being gradually defined [35-36] but has not yet been determined under a variety of conditions. As determined by vapor phase chromatography, the number of acids normally present in human plasma is large. It includes the saturated acids from  $C_{16}$  to  $C_{22}$  and significant amounts of long chain polyethenoid acids greater than  $C_{18}$  in length. Imitic and oleic acids make up the largest component. Short term feeding experiments indicate that the plasma UFA are not greatly affected by the composition of the fat in the diet [37]. Their composition resembles the depot fats [37] but is somewhat higher in certain polyethenoid.

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#### Carotenoids

Carotenoids are aliphatic or alicyclic pigments usually containing 40 carbon atoms (8 isoprene units). Both hydrocarbon (carotenes) and

oxygenated carotenoids (xanthophylls) are present in human plasma and tissues. These nonpolar compounds depend upon solubility in fats or protein binding for transport and in plasma are found mainly with the low density lipoproteins. Hyperlipidemia is commonly accompanied by hypercarotenemia. This is conceivably because of increased absorption or mobilization of carotenoids as the amounts of lipoprotein vehicle in plasma are increased. Carotenoids contribute the yellow color to xanthomas and atheromas that are frequently associated with hyperlipidemia. Among the more recent chemical studies of carotenoids in human tissues are those of Blankenhorn [44a]. A single case of hypercarotenemia attributed to an inborn defect in conversion of carotenoids to vitamin A has been reported [44b].

### *Lipoproteins*

The lipids in plasma and other extracellular fluid are solubilized and made available for transport and biochemical reactions by virtue of their linkage with protein. Except for UFA, which bind primarily to albumin, all the other lipids in plasma are bound to certain of the plasma globulins in the form of lipoproteins. The structural specificity and unique metabolic properties of these macromolecules appear to warrant considering them distinct biochemical entities.

At present lipoprotein analyses do not add essential information beyond that obtained by chemical determination of blood lipids in diagnoses of the hyperlipidemic states. It has not been excluded, however, that the biochemical lesions in certain hyperlipidemias might be defects in lipoprotein metabolism as distinguished from defects in metabolism of only the lipid moieties.

The initial discovery of a lipid protein complex in plasma was made by Macheboeuf in 1929 [45]. As a result of impressive effort by many investigators, a broad spectrum of lipoproteins has been discovered in extracellular fluid. Present understanding of these lipoproteins is derived mainly from the chemical composition and metabolism of certain groups of molecules as rather arbitrarily defined by the methods used to isolate them.

**Isolation.** The ordinary extraction of plasma with most fat solvents destroys the stability of lipoproteins by either dehydrating the molecules or denaturing the protein moieties. This makes it necessary to isolate the lipoproteins by gentler methods based on certain of their physical properties. These methods include electrophoresis, precipitation in media of high ionic strength, and ultracentrifugation. The most versatile of the present methods utilize the low densities of the lipoproteins to separate them from the other plasma proteins and to effect separation within the lipoproteins themselves.

Gofman and his colleagues [46-48] have pioneered in developing

methods for using the ultracentrifuge to segregate lipoproteins according to their densities. Several useful modifications of their technique are available for separating broad groups of lipoproteins in quantities sufficient for chemical analysis [49-50]. In these methods, concentrated salt solutions are added to plasma to achieve a desired density and the solution is centrifuged in fields which may exceed  $100,000 \times$  gravity. In this way the lipoproteins lighter than the density of the medium are concentrated at the top of the centrifuge tube and may be selectively removed for quantitation. This is accomplished by actual analyses of lipid and protein content or by recentrifugation in an analytical ultracentrifuge. The latter is equipped with an optical system through which concentration gradients created by floating lipoproteins may be seen and photographed. The concentrations of these subgroups or density classes are obtained by measuring the area beneath the migrating peaks.

According to the method of Lindgren, Elliott, and Gofman [46], the flotation rates of lipoproteins of density less than 1.063 are assigned values expressed in  $S_f$  units (Svedberg units of flotation in  $10^{-13}$  cm/cc/dyne/gm). The  $S_f$  values of lipoproteins vary directly with their speed of flotation, a property inversely dependent upon the density. So-called standard  $S_f$  units [47] refer to values corrected for temperature and for the effects of overall lipoprotein concentration on the flotation rates of a given class.

It is most likely that a single lipoprotein system exists for all extracellular fluid even though the concentrations of most lipoproteins tend to be somewhat higher in plasma than in lymph. The lipoproteins represent a density spectrum from about 0.9 to 1.2 gm per ml. Within this spectrum they may be most conveniently divided into three large groups, each capable of some further subdivision. These major groups are the high density lipoproteins and low density lipoproteins (according to the Gofman terminology) and the chylomicrons.

**High density Lipoproteins.** The high density lipoproteins isolated by ultracentrifugation between densities 1.21 and 1.063 are also known as  $\alpha_1$  lipoproteins because of their migration with the  $\alpha_1$  globulins on electrophoresis. In the analytical ultracentrifugal technique of deLalla et al. [47] they are not assigned  $S_f$  values but have been subdivided into groups designated the HDL<sub>1</sub>, HDL<sub>2</sub>, and HDL<sub>3</sub> lipoproteins which increase in density in this order. Concentrations of these subgroups change independently under certain conditions [48].

**Low density Lipoproteins.** Low density lipoproteins are floated in the ultracentrifuge at density 1.063. As indicated above, these lipoproteins may be assigned  $S_f$  values when the analytical ultracentrifuge is used. Their concentrations are often measured in convenient groups such as for molecules lying between  $S_f$  0 to III, 10 to 20, 20 to 100, and 100 to 400. Low density lipoproteins of  $S_f$  0 to 12 which float between densities

1 063 to 1 019, migrate with the  $\beta$  globulins. On starch block electrophoresis those above  $S_f$  12 migrate with  $\alpha_2$ -globulins [51]. Hence the terms  $\beta$  lipoproteins and  $\alpha_2$ -lipoproteins are often used in connection with low density lipoproteins. Above  $S_f$  400 the low density lipoproteins merge without a sharp line of demarcation into the third arbitrary group of lipoproteins the chylomicrons.

**Chylomicrons** Chylomicrons as they were first termed by Gage [52] are large (0.5 to 1.5  $\mu$ ) particles of emulsified fat associated with small but probably significant amounts of protein. Normally present in lymph and plasma for only a few hours after the ingestion of fat they can be abundantly present in hyperlipemic conditions in the postabsorptive state.

Since particles above about 0.1  $\mu$  scatter light increased concentrations of chylomicrons or the large low density lipoproteins cause lactescence in plasma. These lipoproteins are also rich in triglyceride and lactescence (or hyperlipemia) invariably means hyperglyceridemia. Light scattering is also a function of particle size and one cannot, therefore make a direct correlation between the degree of lactescence and the chemically determined concentrations of glycerides.

**Other Plasma Lipoprotein** A small amount of lipid soluble phosphorus is present in the plasma fraction of greater density than 1.21 [49]. It is not certain whether this represents lipoprotein of very high density or phosphoprotein. It has recently been reported [23] that as much as 8 per cent of the plasma phospholipid may be present in this fraction about half as lysolecithin. This is associated with other fatty acid ester but little or no cholesterol. The significance if any, of this lipid in essential hyperlipidemia has not been explored.

**Lipoprotein Composition** Table 16-2 illustrates the relationship between composition and density of the lipoproteins. In normal individuals about one third of the plasma cholesterol is present in high density or  $\alpha_1$  lipoproteins. Most of the rest is present in low-density lipoproteins of  $S_f$  0 to 100. It will be observed (Table 16-2) that the proportion of cholesterol to phospholipid is much greater in the low density lipoproteins than in the high density lipoproteins. Essential hyperlipidemia is always characterized by elevation of one or more low density lipoprotein fractions and often by an elevated cholesterol to phospholipid ratio.

Specific differences in composition within the chemical classes of lipids in different lipoproteins need to be further explored. The lipoproteins of density < 1.063 contain a higher molar ratio of sphingomyelin to lecithin and lower ratios of cephalin to sphingomyelin and lysolecithin to lecithin [54-55] compared with the high density lipoproteins.

The nature of the lipid protein bond is not certain but it is loose enough to permit cholesterol and phospholipid molecules to transfer

TABLE 10-2 COMPOSITION OF SEVERAL FRACTIONS OF HUMAN SERUM LIPOPROTEIN

| Fraction                 | Density     | $S_z$<br>(approximate) | Per cent cholesterol |       | Composition  |           |         |       |
|--------------------------|-------------|------------------------|----------------------|-------|--------------|-----------|---------|-------|
|                          |             |                        | Free                 | Ester | Phospholipid | Glyceride | Protein | FC/TC |
|                          |             |                        |                      |       |              |           |         |       |
| Chylomicrons             | <1.006      | >400                   | 3.1                  | 0.1   | 7.1          | 81.3      | 9.5     | 0.47  |
| Low-density lipoprotein  | <1.019      | 12-100                 | 6.0                  | 16.2  | 17.9         | 51.8      | 7.1     | 0.37  |
| Low-density lipoprotein  | 1.010-1.063 | 0-19                   | 8.5                  | 49.3  | 23.6         | 5.1       | 90.5    | 0.25  |
| High-density lipoprotein | 1.063-1.21  |                        | 2.3                  | 18.5  | 20.9         | 4.6       | 47      | 0.17  |

Density of the medium in which the lipoproteins are floated in high centrifugal fields

Note: FC = free cholesterol TC = total cholesterol PL = phospholipid

Source: J. H. Bragdon et al. (55) obtained by chemical analysis of fractions isolated in the preparative ultracentrifuge



readily from one lipoprotein to another [56, 57] Exchange of lipid also takes place rapidly between erythrocytes and plasma lipoproteins [58] Hence lipids may well enter tissues without taking the entire lipoprotein molecule with them

In contrast to the lipids the protein portions of the lipoproteins are not all exchangeable As determined by the chemical and immunochemical findings of many workers summarized elsewhere [38] the peptide residues of groups of lipoproteins of different densities are not the same 'Inter-conversion' of lipoproteins labeled in the protein moiety has been observed within the low density lipoproteins [59] but not between low density and high density lipoproteins [59-60] These two groups of lipoproteins may be quite unrelated in their physiologic function The relationship of chylomicrons to the other lipoproteins is not certain The small amounts of protein associated with these large particles (Table 16-2) include polypeptides identical with those in the high density lipoproteins [61]

It has been proposed that chylomicrons might be shorn of lipid and give rise to the other lipoproteins present in plasma [62] Defects at various stages of such transformations could then account for accumulation of abnormal amounts of various lipoproteins characteristic of different types of hyperlipidemia The evidence presently available does not adequately support this attractive hypothesis [38] although it may be valid for part of the lipoprotein spectrum [59] Several preliminary reports have described synthesis of lipoprotein by the liver [63-65] Since this organ contributes most of the cholesterol and phospholipid to the plasma this is a logical development Incorporation of labeled amino acids into chylomicron proteins by isolated dog intestinal mucosal cells has also been observed [66] The mechanisms and sites of lipoprotein formation may be keys to understanding hyperlipoproteinemia, but acquisition of this knowledge is confronted with many unusual technical difficulties

**Xanthomatosis** Essential hyperlipidemia is commonly recognized through one of two manifestations xanthomas or atherosclerosis Both involve accumulation of lipid in tissues and the mechanisms responsible have been under study and debate for many years No attempt will be made here to review the voluminous literature pertaining to these problems The weight of current evidence indicates that in the presence of hyperlipidemia xanthoma formation and accelerated development of atherosclerosis are secondary phenomena Abnormal amounts of lipid are taken up from extracellular fluid either because the lipids are there in excessive concentrations or because the factors responsible for their solubilization permit the equilibrium to shift in the direction of tissue deposition

The location and appearance of xanthomas in hyperlipidemia depend upon among other things the nature of the abnormal blood lipid pattern

Four or five xanthoma types are distinguished and will be mentioned in subsequent clinical descriptions of these syndromes. There are xanthelasma, tendon, tuberous and plane xanthomas common to essential hypercholesterolemia, and eruptive xanthomas which appear only in severe hyperlipemia. Detailed descriptions of xanthomas appear in all standard dermatology texts and in Thannhauser's excellent monograph on the lipidoses [21]. A helpful clarification of the variable terminology used has been provided by Crocker [67]. Literature concerning arcus senilis or corneal arcus [68], an ophthalmologic change sometimes associated with hypercholesterolemia, has been reviewed by Klatzkin [69].

## ESSENTIAL FAMILIAL HYPERLIPIDEMIA

Hyperlipemia, when defined as an elevation of plasma glycerides above 100 to 150 mg per 100 ml in the fasting state, is not rare, although large scale studies fixing the normal limits in any population have not been made. When defined as it usually is, as sufficient hyperglyceridemia to produce visible lactescence in plasma, hyperlipemia is more uncommon, but still not a rare phenomenon. Since all hyperlipemia, except that seen postprandially, is essential, in that the biochemical mechanism is not understood, it is currently a very difficult task to classify satisfactorily the causes of this manifestation. Several of the causes, but certainly not all, probably involve inherited defects in lipid or lipoprotein metabolism. Of these, hyperlipemia is associated mainly with the trait defined subsequently as essential familial hypercholesterolemia. In addition, there is a smaller group of cases, probably including more than one abnormal trait, in which severe hyperlipemia is the outstanding inheritable feature. These are grouped in the trait which has come to be known as essential familial hyperlipemia.

### HISTORICAL ASPECTS

The historical development of essential familial hyperlipemia begins in 1932, when the first case was reported by Burger and Grutz [70]. Subsequently more than forty cases have been reported in which the outstanding feature has been severe hyperlipemia, closely related to the intake of dietary fat. The hyperglyceridemia has usually been accompanied by elevated concentrations of cholesterol and phospholipids, but of a much less significant degree. In 17 cases involving 11 pedigrees, the hyperlipemia was found to be familial [71-81]. The authors reporting the first such familial case, Holt, Aylward and Timbres [71], coined the term *idiopathic familial hyperlipemia*. Their patient, a 12-year-old girl, was also the first to demonstrate the rare association of fever and abdominal pain developing when the hyperlipemia became severe (total lipids about 8 gm per 100 ml). As the fever declined, so did the hyperlipemia. The liver and spleen grew larger as the hyperlipemia decreased.

and the authors considered that the excess fat in the blood may have been taken up by these organs

Although not all the above forty-odd cases were shown to be familial they shared many common features. In many there were associated eruptive xanthomas, hepatomegaly, and bouts of abdominal pain. In a very few glycosuria was observed but not diabetes. Very infrequently pancreatitis developed after xanthomas or hyperlipemia had been present for some time [73, 77, 82]. In none did the initial report mention significant cardiovascular disease, xanthelasma, tuberous, plane, or tendon xanthomas, or corneal arcus.

In the past few years many cases of hyperlipemia have been reported as "essential hyperlipemia" which differ clinically from the more homogeneous features of the above cases. These include many of the cases reported by Lever [83], Malmros [84], Schettler [85] and their colleagues Adlersberg [86] and others [80, 87]. Practically all these patients have been adults. Many have had xanthelasma, tuberous or even tendon xanthomas, and evidence of severe coronary artery disease. Some have had moderately severe diabetes [88, 89]. In a number of the relatives of probands hyperlipidemia has been present but usually hyperglyceridemia has been slight and the picture indistinguishable from an essential familial hypercholesterolemia.

Borrie [90], analyzing this entangled problem in 1957, concluded that most of the patients reported by Lever and Malmros had essential hypercholesterolemia and associated hyperlipemia. He employed the term 'mixed cases' for that confusing group in which either diagnosis could reasonably be made. In the third edition of his book Thannhauser [91] also recognized a group of patients with hyperlipemia in which he considers the primary disease to be essential hypercholesterolemia.

Further confusion is offered by the recent interesting study of Hirschhorn et al. [91] in which hyperlipemia was diagnosed in Swedish students by determination of the serum optical density in the fasting state and during oral fat tolerance tests. Although their report does not describe studies in the families of the probands, the authors concluded that the incidence of "familial hyperlipemia" in this population was between 2 and 3 per cent. They define this condition as an inherited disease associated with early onset of coronary atherosclerosis and thought to be due to a single gene difference causing a defect in the lipemia clearing system.

It is obvious that clinical distinction between the syndromes of essential hyperlipidemia has become increasingly arbitrary when significant hyperglyceridemia is also present. It is necessary to adopt one of two assumptions. Either essential hyperlipidemia is only one basic disease with many variants in the clinical pattern, or there are several basic disorders with overlapping clinical features. An argument for the possibility of a single

le ion associated with an entire spectrum of abnormal lipid and lipoprotein patterns is provided by the variable manifestations of hyperlipidemia which may be seen in the nephrotic syndrome. On the other hand the extreme manifestations of the clinical syndromes defined below and in the following section as essential familial hyperlipidemia and essential familial hypercholesterolemia are very different and the majority of patients who appear to fall into one of these two groups do not show evidence of converting from one syndrome to the other. The concept of multiple disorders is also supported by the observation of basic differences within the group of patients who have the essential hyperlipidemia indicating the presence of several varieties of this syndrome alone.

Until better means of biochemical differentiation become available the classification of patients with lactescent serum not secondary to other disease as having essential hyperlipidemia is no doubt proper as long as it is recognized that semantic unity may hide biochemical heterogeneity.

Because of present uncertainty the diagnosis and discussion of essential familial hyperlipidemia will be somewhat arbitrarily restricted here to that small group of patients whose hyperlipidemia and other manifestations resemble the dramatic features of the cases described by Burger and Grutz [70] and Holt et al [71]. It is fully realized that lesser expression of the responsible abnormal traits may be present possibly in heterozygotes and account for many more or less dramatic cases not included in the discussion. Although the hyperlipidemia in this syndrome appears to be due primarily to defective removal from the plasma of exogenous fat in the form of chylomicrons the possibility of defective endogenous glyceride metabolism will also be considered.

### CLINICAL FEATURES

The clinical features of 17 cases of essential familial hyperlipidemia in which familial occurrence has been demonstrated [71-81] appear in Table 16-3. About 25 other cases superficially similar but lacking known familial occurrence have been reported. Most of these 25 cases are usually included in consideration of this syndrome and specific references to them appear in several reviews [83-92-93]. That their inclusion is probably justified may be seen by comparison of their clinical features also tabulated in Table 16-3.

There is a tendency for cases not shown to be familial to be detected later in life and several [21] have had some evidence of poor carbohydrate tolerance. The latter has never been reported in a familial case unless pancreatitis was also present. Largely because of this Thannhauser has arbitrarily divided this syndrome into childhood cases and adult cases with occasional glycosuria. For reasons discussed below the author of this chapter has excluded a number of his own cases with moderate to

TABLE 16-3 CLINICAL FEATURES OF ESSENTIAL FAMILIAL HYPERLIPIDEMIA

|                     | Age at onset (yr) |     | Sex |   | Elevated serum triglyceride (mg/100 ml) | Elevated serum phospholipid (mg/100 ml) | Abdominal pain | Eruptive xanthomas | Plasma lipid concentration (mg/100 ml) |     |
|---------------------|-------------------|-----|-----|---|---|---|----------------|--------------------|--|-----|
|                     | <1                | >21 | M   | F |   |   |                |                    | TG                                     | TC  |
| Known family (17)   | 2                 | 5   | 13  | 4 | 10                                      | 10                                      | 8              | 4                  | 3100                                   | 400 |
| Unproved family (6) | 14                | 1   | 70  | 6 | 17                                      | 16                                      | 11             | 11                 | 4700                                   | 484 |

NOTE: Only 1 of 14 children has been examined by the author. The values for triglyceride (TG) and total cholesterol (TC) are the mean values.

severe hyperlipemia and mild glucose intolerance from this classification. They may well prove to represent a separate syndrome.

### Detection

The syndrome is likely to be discovered incidentally with the finding of milky serum on routine examination or, less frequently, during clinical evaluation for hepatic or splenic enlargement or because of sudden development of eruptive xanthomas. Rarely the hyperlipemia will be observed for the first time by an alert physician examining a patient who is in an acute 'surgical emergency' with severe abdominal pain and signs of peritoneal irritation.

The age of detection has varied from a few months to middle age. About half the known familial cases were detected before age 6 and there undoubtedly is a tendency for childhood cases to have more severe manifestations. Many patients appear in good health and may have no other abnormal finding than hyperlipemia. It is probable that the syndrome is not so rare as the number of case reports suggests.

### Plasma Lipids

The serum is characteristically laetescens, varying from moderate turbidity to the appearance of cream (which indeed it may approximate in total lipid content). Plasma lipid analyses for four familial cases studied in the author's clinic are shown in Table 16-4. Since the levels tend to fluctuate greatly in any one patient, the values associated with the maximum total lipid concentrations obtained in a series of analyses are presented. Total lipids of 18 gm per cent have been reported [94]. The important features are triglyceride concentrations twenty to forty times normal, associated with cholesterol and phospholipid concentrations increased by only two- to threefold. More important than the absolute concentrations is the effect of dietary fat restriction. This always causes

a rapid fall in all elevated plasma lipids the relative proportions between the several lipids remaining about the same. The hyperlipemia sometimes seen in essential familial hypercholesterolemia may respond to the same treatment but the cholesterol and phospholipids will often remain disproportionately elevated.

The frequent finding of a lower percentage of esterified cholesterol (Table 16-4) is no doubt related to a higher percentage of free cholesterol in the triglyceride-rich lower density lipoproteins characteristic of severe

TABLE 16-4 PLASMA LIPID ANALYSES IN FOUR PATIENTS WITH ESSENTIAL FAMILIAL HYPERLIPIDEMIA

| Patient | TL<br>mg/100 ml | TG<br>mg/100 ml | TC<br>mg/100 ml | PL<br>mg/100 ml | EC<br>per cent | TG/TC |
|---------|-----------------|-----------------|-----------------|-----------------|----------------|-------|
| J P     | 2 685           | 2 014           | 234             | 337             | 55             | 8.6   |
| I P     | 3 510           | 2 916           | 239             | 281             | 57             | 12.4  |
| P P     | 2 110           | 4 61            | 363             | 314             | 43             | 17.0  |
| J L     | 580             | 4 037           | 668             | 517             | 63             | 6.0   |
| Mean    | 1 950           | 3 339           | 316             | 376             | 5              | 9.8   |
| Normal  | 150             | 125             | 240             | 250             | 70             | 0.4   |

Upper limits of normal for all age groups in the author's laboratory

NOTE: TL = total lipids TG = triglyceride TC = total cholesterol PL = phospholipid EC = esterified cholesterol

hyperlipemia (Table 16-2) and is not an indication of hepatic disease. Conventional liver function test results with the possible exception of a slightly elevated thymol turbidity are normal in essential familial hyperlipemia. The presence of turbidity often makes the thymol test impossible to determine accurately.

In a few patients studied so far [9-81] fasting UFA levels have not been recognizably abnormal.

### Lipoproteins

The lipoprotein changes in essential hyperlipemia are characterized by enormous increases in chylomicrons and low density lipoproteins of high flotation rates. A comparison of the changes in a large number of lipoprotein classes as isolated ultracentrifugally may be seen schematically represented in Fig. 16-2. Reciprocal decreases in high density lipoproteins particularly the HDL<sub>2</sub> fraction and sometimes in S<sub>0</sub> to 20 low density lipoproteins are a common occurrence in severe hyperlipemia regardless of whether the latter is essential or secondary. When the hyperlipemia is reduced the lower concentrations of these lipoproteins are usually but not always restored toward normal.

The lipoprotein findings obtained by electrophoresis vary with the method used. Paper electrophoresis will demonstrate great increases in

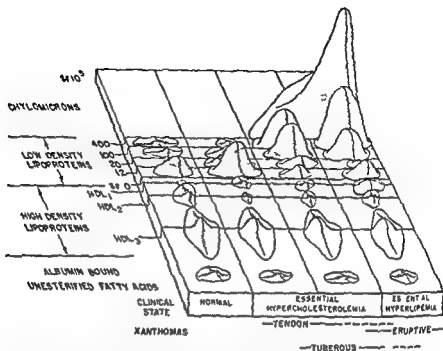


Fig. 16-2. Distribution of lipid in lipoproteins in essential hyperlipidemia. Only the lipid moieties are represented as calculated from the data of Gofman et al [48] from the known lipid composition of the various fractions. For emphasis what is actually a continuous spectrum of changes has been presented in three groups alongside the normal distribution. The presentation does not conform to scale and the indicated association of xanthomas with various lipid patterns is only relative.

the  $\beta$  lipoproteins and a chylomicron tail will appear at the origin. Starch block or moving boundary electrophoresis will reveal increases in the  $\alpha_2$  peak and sometimes in the  $\beta$  peak [95].

### Hepatosplenomegaly

This has been observed in approximately two thirds of the cases discovered before age 6 and in about one-third of those detected later. Only one organ or both may be enlarged. The size of the organs may change greatly and in every case has been reported to decrease with institution of a low fat diet. A possible cause of organ enlargement is accumulation of lipid although Thannhauser [21] disagrees with this hypothesis as far as the liver is concerned. In the single autopsy reported for this disease by Chapman and Kinney [96] Thannhauser and Reichstein found that the liver fat content was not abnormal except for a possible decrease in sphingomyelin. It is noteworthy that this child died of a severe intercurrent infection and the history suggests that it may have been malnourished at the time of death. The liver contained no foam cells.

but they were prominent in the spleen. A case described by Rohn et al [97] in which the diagnosis of essential hyperlipemia cannot be made with certainty also came to autopsy. The patient was a young female discovered to have marked hyperlipemia and hepatomegaly in the eighth month of pregnancy. Phagocytic cells laden with fat were observed in liver and sternal marrow biopsies. She died suddenly at term. The liver was found to contain minimal fatty infiltration but the spleen was almost replaced by foam cells. The cause of death was splenic and portal vein thrombosis.

### *Foam Cells*

In most cases foam cells may be found in biopsies of tissues rich in reticuloendothelial cells. They are usually present in bone marrow and spleen and may appear in liver and lymph nodes. These large histiocytes are 10 to 90  $\mu$  in diameter and contain droplets of lipid which give the cytoplasm a finely reticulated or mulberry appearance. The droplets stain variably with Nile blue and Sudan III [96] but in the author's experience are Smith Dietrich negative. Since the foam cells may very closely resemble the Niemann Pick cell (Chap. 18) in overall appearance this latter staining reaction is of academic interest although the two diseases are easily separated by other considerations.

The foam cells in essential hyperlipemia are identical to those early reported in the livers of diabetic patients [98-99] and no doubt arise by the same mechanism leading to xanthoma formation. Presumably this is a phagocytic reaction of the reticuloendothelial system to the excessive fat in the blood and does not represent any increased synthesis of fat *in situ*. Vacuolization of parenchymal as well as Kupffer and sinusoidal cells has also been observed in biopsy specimens from the liver [74].

### *Xanthomas*

The characteristic skin xanthoma in essential hyperlipemia is of the eruptive type (Fig. 16-3). The lesions characteristically appear when the hyperlipemia is severe and disappear when it is reduced by a low fat intake. There is no predictable triglyceride level or duration of hyperlipemia at which lesions will appear. The author has seen them appear in a patient with plasma triglycerides at only 2,000 mg per 100 ml for a short time and fail to develop in a young patient whose plasma triglycerides averaged 4,000 mg per 100 ml for over 15 years. In several cases [70-71, 100] small vesicles which have broken down and exuded milky fluid have also been described.

By definition a number of cases of severe hyperlipemia with tubercous and tendon xanthomas have been excluded from the series of patients considered to have essential familial hyperlipemia. It cannot be assumed dogmatically however that they never occur. The clinical distinction



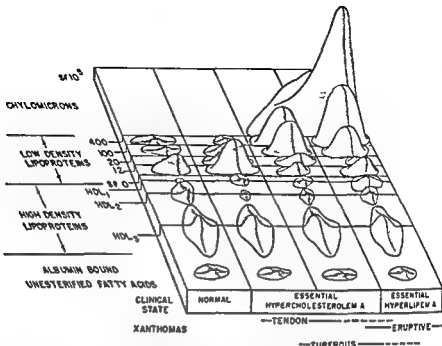


Fig 10-2 Distribution of lipid in lipoproteins in essential hyperlipidemia. Only the lipid moieties are represented as calculated from the data of Gofman et al [48] from the known lipid composition of the various fractions. For emphasis what is actually a continuous spectrum of changes has been presented in three groups alongside the normal distribution. The presentation does not conform to scale and the indicated association of xanthomas with various lipid patterns is only relative.

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It may be associated with spasm rigidity and rebound tenderness leukocytosis and fever There may be vomiting and collapse Other laboratory tests including those for serum amylase or lipase will usually give negative results and only the observation of grossly milky serum may spare the patient a needless laparotomy For excellent descriptions of these abdominal crises see the reports by Holt et al [71] and Bloomfield and Shen on [102]

The attacks of abdominal pain are rarely so far as can be determined at laparotomy associated with abnormality of the pancreas In fact there often is no visible abnormality save possible enlargement of liver or spleen The cause of pain has been thought to be distention of these viscera by uptake of lipid [71] The blood is not necessarily free of hyperlipemia at the height of the pain

### *Pancreatitis*

Poulson [73] and Kennedy and Collett [82] have reported cases of familial hyperlipemia in which eruptive xanthomas or hyperlipemia have been observed well in advance of an attack of abdominal pain which proved at laparotomy to be associated with unquestionable pancreatitis Klatkin and Gordon [77] have also reviewed this problem and have presented several patients with similar convincing evidence that hyperlipemia can precede pancreatitis They postulated that fat embolization may cause this disorder but this has not been demonstrated experimentally The mechanism is not known It seems unlikely that all cases of acute pancreatitis which is not a rare disease should be due to essential hyperlipemia Why the former is often associated with transient hyperlipemia likewise remains an enigma

### DIFFERENTIAL DIAGNOSIS

The diagnosis of essential familial hyperlipemia depends upon the exclusion of any cause of secondary hyperlipemia and the hyperlipemia associated with essential familial hypercholesterolemia The nephrotic syndrome von Gierke's disease or the rare cases of hyperlipemia associated with myxedema xanthomatous biliary cirrhosis neoplasia hemorrhage and other obvious primary diseases present little difficulty Hyperlipidemia associated with pancreatitis or mild diabetes may offer confusion Unless hyperlipemia is known to have antedated either of these conditions the concomitant diagnosis of essential familial hyperlipemia should not be made Essential familial hypercholesterolemia with hyperlipemia is often associated with tuberous and tendon xanthomas and cardiovascular disease and there is a more pronounced familial involvement The predominantly elevated lipoprotein classes are often low density lipoproteins of high flotation rate rather than primarily chylomicrons This syndrome is discussed more fully further on in this chapter

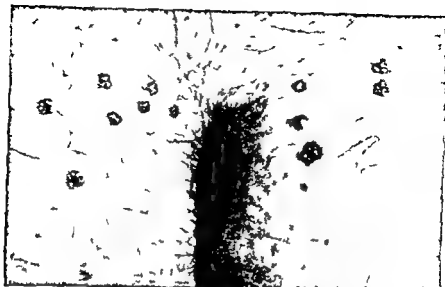


Fig 16-3 Eruptive xanthomas on the buttocks of a patient with essential hyperlipemia. These lesions completely disappeared after 4 weeks of very low fat intake.

between eruptive and tuberous xanthomas (Fig 16-8) is sometimes arbitrary. Both terms have been used in the literature for isolated yellow plaques with an inflammatory base.

### *Lipemia Retinalis*

Lipemia retinalis occasionally offers a basis for detection of severe hyperlipemia. This sign consists of a marked increase in light reflex of the retinal vessels and a pale cast to the fundus. It has been stated [101] that a triglyceride concentration of over 2 000 mg per 100 ml is required for the appearance of lipemia retinalis, but probably this is also a function of the particle size of the lipoproteins present. Lipemia retinalis is no more specific a sign in essential hyperlipemia than is the incidental finding of milky fluid on a pleural tap, for example. Either simply reflects elevated glycerides and very low density lipoproteins in the extracellular fluid.

Dunphy [101] and Falls (in discussing the former's paper), describe several cases with clouding of the cornea or lipid interstitial keratitis in association with hyperlipemia. The exact nature of this symptom is obscure, but it probably is due to some incidental process associated with transudation of lipid-rich lymph.

### *Abdominal Pain*

Slightly less than half the patients with essential hyperlipemia have complained of episodic abdominal pain. Frequently epigastric or mid-abdominal, the pain is often not localized and may radiate to the back.

decreased by higher initial concentrations or by the injection of a previous load of chylomicrons [106]. Although the behavior is said to be characteristic of removal of a substance by the reticuloendothelial system, there is no certain evidence that these cells are more important than parenchymal cells in the removal of chylomicrons. When isolated rat liver is perfused with chylomicrons, more fat droplets have been observed in the parenchymal cells than in the Kupffer cells [107].

The tissues capable of removing chylomicrons include liver, heart, skeletal muscle, and adipose tissue [108]. In vivo studies [108] suggest but have not proved that adequate carbohydrate metabolism is necessary for uptake by adipose tissue. The isolated perfused liver has been shown to be capable of both removing and oxidizing chylomicron triglyceride fatty acids (TGFA) [107], and it is probable that this is true of most other sites of removal. Many of the chylomicron triglyceride fatty acids are oxidized shortly after removal, and it is well established that these incoming fatty acids are readily available for metabolism [57].

There are indications that chylomicrons are degraded during their removal. As chylomicrons leave the blood, the triglyceride fatty acids rapidly reappear in the blood as UFA [109]. It has been estimated that much of the TGFA is directly oxidized without retransport as UFA [34], but hydrolysis may still be a necessary step in the tissue uptake of chylomicron triglyceride.

Granted the close association between hydrolysis and removal of chylomicrons, there are two schools of thought concerning the site of hydrolysis and participation of other plasma lipoproteins in this process.

Observations of labeled chylomicron removal in the rat [111] and the dog [109] suggest that chylomicron triglyceride does not pass through other low-density lipoproteins in plasma during its removal, or if it does, that these other lipoproteins turn over with extremely rapid rates. This implies that at tissue cell interfaces either chylomicrons are removed intact or the triglyceride is hydrolyzed, with removal of some or all of the hydrolysis products by the cells.

The possibility that chylomicrons may escape the vascular system intact is supported by the observations that their removal from the blood is accompanied by the appearance of a small concentration of chylomicrons in the hepatic lymph [112]. Capillary pores in the endothelial wall have been observed by electron microscopy [113]; they suggest a possible means of egress.

On the other hand, it has been suggested that, particularly in man, the degradation of chylomicrons takes place primarily intravascularly. This involves the hydrolysis of triglyceride, with the removal of the product fatty acids as UFA bound to albumin, leaving residual lipoproteins of higher density which may be degraded sequentially. This theory has

## POSSIBLE BIOCHEMICAL DEFECTS IN ESSENTIAL HYPERLIPEMIA

It must be emphasized again that very probably not all cases currently defined as essential hyperlipemia arise from the same metabolic defect. In no case is the exact mechanism understood. Two possible sources for the excessive amounts of glyceride in the plasma exist: (1) defective removal from blood of exogenous glyceride absorbed from the diet; (2) release into the plasma of more endogenous glyceride than can be removed at a rate sufficient to maintain normal concentrations. These two possibilities may be considered in turn, approached first from the standpoint of what is known of the normal mechanisms involved.

*Removal of Exogenous Triglyceride*

**Absorption and Chylomicron Formation** The fat in the diet consists largely of triglyceride of which 30 to 100 gm is consumed daily by the average adult. The mechanisms of digestion of fat and its transfer across the intestinal mucosal cell into the lymph are only partially understood. For detailed analysis of current research in this area, reference may be made to recent reviews by Bergstrom and Bergstrom [103] and Frazer [104].

From work of Bloom et al [105] and Bergstrom and Bergstrom and their colleagues [103] it is known that the fatty acids of chain length  $C_{10}$  and greater are reincorporated into triglycerides during absorption and transported to the blood in the lymph via the thoracic duct. Small amounts of some acids such as stearic are also transported as phospholipid. Very little is transported as unesterified fatty acid or as cholesterol esters.

As the glycerides of acids of longer chain length are reformed in or near the mucosal cells, they appear in the intestinal lacteals as chylomicrons. As the chylomicrons accumulate, the lymph becomes lactescent and the milky chyle is carried to the blood via the thoracic duct. The maximum concentration of glyceride and chylomicrons is usually reached 3 to 4 hr after ingestion of fat. Increased amounts of cholesterol and phospholipid are also carried in lymph with the chylomicrons, and their concentrations in plasma may rise during fat absorption. After the chylomicrons have disappeared, small increases in lipid in higher density lipoproteins may remain for short periods.

**Chylomicron Removal** The mechanisms for removing chylomicrons from the blood are only partially understood. It is not known whether the chylomicrons pass into tissues intact or whether they must first be degraded either at the capillary cell interface or in the blood. It is known that the triglyceride moiety of chylomicrons is rapidly removed from the blood at an exponential rate and that the fractional turnover rate (the fraction of the total amount in blood disappearing per unit time) is

**Factors Influencing Chylomicron Removal** One of the more important factors affecting chylomicron removal appears to be carbohydrate metabolism. Retardation in removal of fat emulsions from the plasma of diabetic dogs has been demonstrated [122]. Recent experiments in human beings have been interpreted as indicating that alimentary lipemia is decreased by factors promoting carbohydrate utilization such as glucose feeding or glucagon administration and increased by factors decreasing carbohydrate utilization such as fasting or epinephrine administration [123]. Carbohydrate feeding has not yet been shown experimentally to increase chylomicron removal and the effect of carbohydrate metabolism on fat absorption has not been adequately explored as a possible explanation of the above phenomena.

**Possible Defects in Chylomicron Metabolism in Essential Hyperlipemia** The concept that in essential hyperlipemia exogenous fat is removed at an abnormally slow rate (so-called retention hyperlipemia [21]) has been supported by some pertinent observations. These include the well established clinical experience that hyperlipemia in such patients is often a direct function of dietary intake of fat. An intake over 30 to 50 gm per day will lead to marked hyperlipemia and in a case such as that of Holt et al [71] to abdominal crises and marked hepatic and splenic enlargement. This latter suggests that removal of chylomicrons by reticuloendothelial phagocytosis becomes significant in extreme degrees of hyperlipemia. A low fat intake diet will always reduce the hyperlipemia. In hyperlipemic members of the P family (see below) fat free diets restore blood lipids to very nearly normal levels in a few days. Havel [124] injected intravenously lipemic plasma from one of these patients into the patient after his blood lipids had been normalized by diet and into a normal control. In the patient the chylomicrons disappeared over a matter of days; in the normal person they were removed in a few hours.

A slower rate of clearance from the blood of fed  $I^{131}$  triolein [125-126] or intravenously injected olive oil or triolein emulsions [127] has been reported in patient with essential hyperlipemia when compared with normal persons or patients with essential hypercholesterolemia without hyperlipemia. Since presence of hyperlipemia may overload the chylomicron clearing mechanism and decrease the fractional removal rate of chylomicrons, experiments designed to prove a basic defect in clearing should properly be conducted with the recipient in a normolipemic state. Only a few experiments such as those reported by Havel [124] and by Crofford et al [126] provide unequivocal evidence of delayed chylomicron removal in essential hyperlipemia.

Enough evidence is available to permit speculation about only three possible sources of the defect in chylomicron metabolism. These are a deficiency in lipoprotein lipase activity, presence of abnormal lipoproteins,

arisen largely from the work of Gofman and his colleagues who have shown that during the clearing of hyperlipemia there occur stepwise increases in low density lipoproteins of progressively greater density and lower flotation rates [38]. Thus "interconversion" has also been reported in human beings following the ingestion of a fat meal [114]. There is no doubt that "interconversion" within certain low density lipoprotein classes may occur physiologically; a defect in such "conversion" has been proposed as the origin of hyperlipemia in the nephrotic syndrome [39].

Although experiments employing labeled phospholipid [115], cholesterol [67] or protein [66] portions of the chylomicrons have shown that these moieties leave the plasma more slowly than the triglyceride portion, it has not been possible to determine whether lipoprotein "skeletons" of higher density are actually left in the blood after departure of the chylomicron triglyceride. This is because both labeled protein and lipid may passively exchange between chylomicrons and other lipoproteins, a process which may mimic actual metabolic "conversion." Thus a defect in "conversion" of chylomicrons in essential hyperlipemia remains hypothetical.

**Lipoprotein Lipase** It is obvious from the foregoing discussion that some hydrolytic enzyme system or systems plays a pivotal role in chylomicron metabolism and no doubt in the metabolism of endogenous triglyceride and UFA. While several lipases have been isolated from adipose tissue, blood and the pancreatic secretion, the lipase considered most seriously to have a role in fatty acid transport is lipoprotein lipase [116]. It catalyzes the hydrolysis of triglycerides in any plasma lipoprotein or in synthetic emulsions provided a small amount of lipoprotein is available for "activation" [116]. When the reaction occurs in plasma the UFA released by the reaction are bound by albumin,  $D_L$  and monoglycerides are also formed [30]. The degradation of the triglycerides is accompanied by fall in the concentration of the larger light-scattering lipoproteins (which are especially rich in glyceride) and the plasma becomes less turbid. Hence the original designation of the enzymatic activity as "clearing factor." Under physiologic conditions very little lipoprotein lipase activity is measurable in plasma in man [117] or the rat [118]. The activity is increased somewhat by fat feeding. From in vitro measurements of activity in human plasma it has been estimated that the hydrolysis taking place would be sufficient to account for only a small part of the hydrolysis necessary to clear normal fat loads. Plasma lipoprotein lipase activity is increased manyfold by the administration of heparin or heparin like substances. The mechanism of this "activation" or "leakage" of enzyme activity into the plasma is not known. It is of interest however that bacterial heparinase can destroy the activity of lipoprotein lipase [119]. Lipoprotein lipase has been isolated from rat heart and adipose tissue [116, 120] but so far not from liver. In fact the latter organ appears to remove lipoprotein lipase activity from plasma [121].

Theoretically any factor interfering with the processes involved in UFA and fatty acid ester metabolism within the cell (see below) or plasma UFA transport may also interfere with chylomicron removal. The author has measured plasma UFA turnover and oxidation of plasma UFA by isotopic means [129] in four nonfamilial cases of essential hyperlipemia without detection of variation from the still crudely defined limits of normal.

**Abnormal Lipoproteins** It has recently been suggested by Schettler et al [80] that chylomicrons in essential hyperlipemic patients may have abnormal chemical composition. Their experimental data, however, are not based on well washed chylomicrons free of possible contamination with other lipoproteins. Plasma chylomicron from the author's patient J. L. (Table 16-4) have concentrations of cholesterol, phospholipid, and glyceride similar to the concentration of chylomicrons isolated from pooled normal plasma. The N-terminal amino acid residues of the chylomicron protein were also similar to lymph chylomicrons obtained from a normolipemic patient. It is to be noted that chylomicrons of the P family members are readily cleared by normal persons.

Abnormally slow hydrolysis of chylomicrons from patients with essential hyperlipemia upon *in vitro* incubation with lipoprotein lipase has been reported [29, 130]. In the second of the three cases Carlson and Olhagen [130] obtained a reduction in plasma glycerides after heparin administration. Their carefully studied patient had several features suggestive of essential familial hypercholesterolemia, especially no fall in a markedly elevated cholesterol concentration after 4 days of fasting. They concluded that the defect in this patient may have been primarily in endogenous glyceride metabolism.

It has already been indicated that decreases in some higher density lipoproteins occur in hyperlipidemia, especially in marked chylomicronemia. The lack of specificity of the changes and frequent reversion toward normal as the hyperlipemia is reduced suggest that this is a secondary phenomenon. It has been suggested that apparent deficiencies of lipoproteins of density  $> 1.019$  in hyperlipemia may be due to a leaching of their lipid by the excessive concentration of chylomicrons [80, 131]. A primary deficiency in high density lipoprotein could theoretically deprive incoming chylomicrons of sufficient protein for stabilization and activation for lipolysis. Observation that clearing of hyperlipemia in patients with essential familial hyperlipemia may still not correct abnormally low concentrations of high density lipoprotein [124] leaves this intriguing problem yet to be solved.

#### ENDOGENOUS GLYCERIDE METABOLISM AND HYPERLIPIDEMIA

With the possible exception of the unusual P family, it has not been shown that the hyperglyceridemia in essential familial hyperlipemia



or abnormal carbohydrate metabolism secondarily affecting the metabolism of glyceride. It will be more convenient to consider the last possibility later with a discussion of endogenous glyceride metabolism.

**Lipoprotein Lipase Deficiency** Reference has already been made to the discovery by Havel [124] of three hyperlipemic siblings whose plasma contains abnormally low lipoprotein lipase activity after injection of up to 100 mg heparin intravenously (the P family). Enzyme activity is negligible as measured by changes in optical density or in UFA or glycerol concentrations when their postheparin plasma is incubated with their own chylomicrons or artificial triglyceride emulsions. Enzyme activity is present after much smaller doses of heparin in the one other familial case the author has studied (J L, Table 16-4) and in other cases of 'essential hyperlipemia' in which examination for presence of this enzyme in plasma has been reported. A possible exception is the brief mention by Crofford et al [126] of an abnormal heparin response in a hyperlipemic patient.

The paucity of enzyme in the plasma of the P family can only be assumed to represent a tissue deficiency interfering with hydrolysis and delaying chylomicron removal. Since activity of this enzyme has not yet been measured in human tissue it has not been possible to test for deficiency in the hyperlipemic members. It is very probable however that this family represents a rare subgroup of essential familial hyperlipemia and possibly a unique mutation. It is interesting that these siblings (Fig 16-5) who refuse to restrict their fat intake, have had thirty to fiftyfold elevations in glyceride concentrations (Table 16-4) for at least 11 years and probably since birth. One had a 3 day bout of abdominal pain with transient splenomegaly for the first time at age 23. None has any evidence of atherosclerosis.

Standardized measurements of plasma lipoprotein lipase activity following heparin have not been made in a comparison study of essential familial hyperlipemic patients and normal persons matched for age and sex. It is essential that such studies employ UFA or glycerol production rather than optical clearing for measurement of activity. A relative deficiency in enzyme response to heparin has therefore not been excluded in other patients with this syndrome. Evidence has already been presented indicating that if lipoprotein lipase has a physiologic role in chylomicron removal the activity of the enzyme in tissues is most likely more important than in plasma. Adequate assay of lipoprotein lipase activity or of heparin or its analogues has not been made in human tissues.

No inhibitor of lipoprotein lipase activity is present in the plasma of the P family [124]. Lever and Klein [128] have reported inhibition of the enzyme *in vitro* following addition of hyperlipemic plasma obtained from patients with essential hyperlipemia or von Gierke's disease. Their results suggest a nonspecific factor which needs to be further investigated.

have been reported abnormal in a series of patients with "essential" hyperlipemia or hypercholesterolemia [132]. Recent interesting observations indicate that hyperglycemia may depend upon the degree of hyperlipemia [126].

Most patients with unequivocal essential familial hyperlipemia have had a normal glucose tolerance. Thannhauser has emphasized that insulin did not affect the blood lipids in his patients with glycosuria [21]. This was likewise true for patient A. S. in Table 16.5. The pathways for metabolism of fat and carbohydrate are so intertwined however that it is not surprising to find abnormalities in both in hyperlipidemia. What is known of these interrelationships offers a few furtive clues to the causes of essential hyperlipidemia.

#### SYNTHESIS OF GLYCERIDES AND GLYCEROPHOSPHATIDES

The scheme presented in Fig. 16-4 is that suggested by Kennedy [133] who with his coworkers is primarily responsible for discovery of the essential role of the cytidine phosphatides as essential cofactors in these pathways. It is apparent that once the common intermediate 1,2 diglyceride has been formed the reactions may proceed to the synthesis of either triglyceride or glycerophosphatide. This relationship explains some observations of increased phospholipid turnover at times when the major process is net synthesis of triglyceride.

Although it is assumed that lipoprotein lipase activity in adipose tissue may be the physiologic mechanism for releasing UFA, it is unlikely, as Kennedy has pointed out [133], that reversal of lipolysis, a low energy reaction, accounts for much net synthesis of adipose tissue triglycerides. This is true despite the finding that labeled acids are incorporated into glycerides in simple equilibrium experiments catalyzed by lipoprotein lipase [134].

The net synthesis of triglycerides involves the intermediate formation of phosphatidic acid and 1,2 diglyceride. ATP and acyl CoA are essential for the steps [133], as is available glycerol, and it is logical to assume that a major share of the latter comes from glucose degradation. The major role of carbohydrate metabolism in supplying both parts and energy for glyceride and glycerophosphatide synthesis is an important example of interdependence of carbohydrate and lipid metabolism.

In any tissue the metabolism of triglycerides must also be intimately related to the metabolism of fatty acids themselves. Recent reviews of progress in this area include those of Lehninger [135] and Langdon [136]. Probably the enzymes necessary for synthesis of glycerides exist in all tissues capable of synthesizing fatty acids. Two of the most active in this capacity are the liver and the adipose tissue. Shapiro [137] has recently reviewed the evidence indicating that at least a large part of the adipose tissue triglycerides is synthesized *in situ*. Fatty acid synthesis

depends entirely upon exogenous fat intake. Extremely low fat diets have rarely been maintained long enough to eliminate endogenous glyceride as a source of hyperlipemia.

The author has observed at least six patients with rather severe "essential hyperlipemia" which is not obviously dependent upon fat intake. This is indicated in Table 16-5 where results of feeding isocaloric formula

TABLE 16-5 THE EFFECTS OF VARYING FAT INTAKE AND INSULIN IN ATYPICAL ESSENTIAL HYPERLIPEMIA

| Patient      | Day | Daily fat intake                        | TG<br>mg/<br>100 ml | TC<br>mg/<br>100 ml | FC<br>mg/<br>100 ml | PL<br>mg/<br>100 ml |
|--------------|-----|---|---------------------|---------------------|---------------------|---------------------|
| N C (age 44) | -20 | c 100 gm                                | 8100                | 1118                | 474                 | 1242                |
|              | 0   | Begin 5 gm                              | 5776                | 610                 | 210                 | 10                  |
|              | 21  | Begin 100 gm (corn oil)                 | 763                 | 176                 | 68                  | 281                 |
|              | 29  | Begin 5 gm                              | 817                 | 165                 | 64                  | 761                 |
|              | 36  | Begin 100 gm (coconut oil)              | 604                 | 163                 | 62                  | 251                 |
|              | 42  | Begin 100 gm (butter fat)               | 58                  | 172                 | 63                  | 259                 |
|              | 48  | Begin 5 gm                              | 875                 | 229                 | 85                  | 334                 |
|              | 62  | End 5 gm                                | 255                 | 130                 | 36                  | 190                 |
| A S (age 40) | 0   | Begin 5 gm                              | 1074                | 353                 | 118                 | 404                 |
|              | 11  | Begin 100 gm (corn oil)                 | 1047                | 291                 | 103                 | 381                 |
|              | 21  | End 100 gm                              | 453                 | 248                 | 60                  | 261                 |
|              | 0   | Regular inulin 15 units twice daily     | 523                 | 314                 | 80                  | 340                 |
|              | 7   | End regular inulin 15 units twice daily | 665                 | 363                 | 103                 | 3                   |

NOTE: TG = triglyceride TC = total cholesterol FC = free cholesterol PL = phospholipid Day 0 and 0 indicate the start of specific experimental periods

diets to two such patients are presented. Both these patients were obese adult males who also had occasional glycosuria, fasting blood sugar levels of 100 to 120 mg per 100 ml and moderately abnormal glucose tolerance test results. A family history of hyperlipidemia was not elicited but no relatives were available for analyses. Subsequent reduction to ideal body weight has left both patients with nearly normal glucose tolerance and glyceride concentrations not over 200 mg per 100 ml unaffected by isocaloric fat intake.

These cases are consistent with hyperlipemia of endogenous origin related to abnormal carbohydrate metabolism. The association of reasonably controlled diabetes with persistent hyperlipemia has been reported in a number of patients [89]. Glucose and insulin tolerance test results

concentrations occur when intake of food not necessarily fats exceeds caloric demand. These hyperlipemias of feast or of famine have not been shown experimentally to involve increased *net* transport of fatty acids as glycerides but they probably do. They must both be interpreted in the light of what is known about the capacity for transport of fatty acids in plasma as UFA. This transport system with its complementary relationship to carbohydrate metabolism is also linked in comparably to glyceride metabolism.

When carbohydrate utilization is diminished below some critical level UFA pours into the plasma and is taken up by tissues like the liver and muscle to fill the need for oxidizable substrate. As measured by isotopic means this turnover of plasma UFA may be even greater than the caloric demand [41, 129]. It is conceivable that excess UFA may be converted to glyceride which spills back into the plasma. It is not excluded of course that fatty acids may leave the depots directly as glyceride perhaps in response to some stimulus other than need for substrate and merely pile up in plasma having exceeded the capacity of other tissue to remove them. There is good evidence that whatever the form in which they are initially released the elevated fatty acids have their origin in adipose tissue [41]. Adipose tissue may also be reluctant to take them back in the absence of adequate carbohydrate utilization [108].

When carbohydrate utilization is normal and supply exceeds demand some of the excess may be converted to glyceride. Hyperlipemia may result from the transport of this glyceride to adipose tissues for storage. That adipose tissue is capable of synthesizing its own glycerides from carbohydrate [137] does not weaken this hypothesis. This hyperlipemia of caloric excess would of course be exaggerated in patients with any defect in chylomicron metabolism.

### *Humoral Factors*

Several humoral factors have been reported which may cause fat mobilization and sometimes hyperlipemia. These include factors isolated from the anterior pituitary [138, 139] from the blood of cortisone-treated animals or extracts of the posterior pituitary [140] or from the urine of fasting human beings [141]. Epinephrine causes hyperlipemia in certain species but the relationship of this action to effects of epinephrine on the adrenal cortex and on carbohydrate utilization is yet to be determined. Glucocorticoids have been shown to be necessary for production of hyperlipemia in diabetic rats deprived of insulin [142]. The hyperlipemia coincides with the development of hyperglycemia and ketosis. The relationship of emotional stress to hyperlipemia has been little explored although transient elevation of lipid predominantly cholesterol in patients under emotional strain has been reported [143].

None of the general understanding of the interrelationships of car

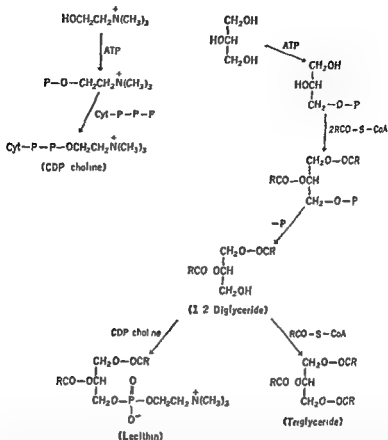


Fig 16-4 The relationship between phospholipid and triglyceride synthesis (after Kennedy [133]) illustrating the pivotal position of 1,2 diglycerides. The reactions for formation of CDP choline (cytidine diphosphate choline) and lecithin have been shown to be identical for the formation of CDP ethanolamine and cephalin (phosphatidylethanolamine).

is dependent upon carbohydrate metabolism as a source of adequate TPNH [136].

#### REGULATION OF ENDOGENOUS TRIGLYCERIDE CONCENTRATIONS IN PLASMA

##### Carbohydrate Metabolism

In the absence of fat ingestion the glyceride concentrations are not negligible being of the order of 100 mg per 100 ml after 16 hr of fast. With continued fasting triglyceride concentrations do not tend to diminish but are on the contrary often increased. The principal lipoproteins which may be elevated include chylomicrons and it is assumed they are formed at sites other than the intestine. Conversely increased glyceride

concentrations occur when intake of food not necessarily fats exceeds caloric demand. These hyperlipemias of feast or of famine have not been shown experimentally to involve increased *net* transport of fatty acids as glycerides but they probably do. They must both be interpreted in the light of what is known about the capacity for transport of fatty acids in plasma as UFA. This transport system with its complementary relationship to carbohydrate metabolism is also linked inseparably to glyceride metabolism.

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bohydrate and fat metabolism nor any of these isolated data on humoral factors, has yet been translated into specific mechanisms regulating either endogenous or exogenous triglyceride metabolism. Until this has been accomplished for the normal state any defective enzyme systems which may lie at the root of essential hyperlipemia will not be understood.

## GENETICS

### *Mode of Inheritance*

Adequate data are not available to ascertain the mode of inheritance of essential familial hyperlipemia. Not only have relatives often not been examined but reports of blood lipid findings have been inadequate. It cannot always be ascertained whether the hyperlipidemia seen in affected relatives is even of the same type as that in the proband.

It has been stated by Hirschhorn et al [91] that "familial hyperlipemia" is a dominant trait in which the homozygous phenotype includes hepatosplenomegaly, abdominal pain and other features shown in Table 16-3 and the heterozygous a tendency toward hyperlipidemia and atherosclerosis. This inference is derived by the authors from the single case report of Holt and coworkers [71]. While this supposition may prove ultimately to be correct it is not yet supported by sufficient case analyses and is rendered especially tenuous by the present difficulty in defining the trait or traits involved.

Of the cases selected here for presentation as essential familial hyperlipemia 10 pedigrees were known to have been affected more than once. One parent of the proband was also involved in three instances [71, 74, 77]. In one of these a maternal aunt in addition to the mother was said to have had elevated blood lipids. In the remaining 7 pedigrees affected individuals have been reported only in a sibship but the parents were specifically stated or known to have been examined and found to be normal in only 2 of these pedigrees.

The pedigree of the Negro P family is shown in Fig. 16-5. The mother and father have slightly elevated total cholesterol concentrations about 260 mg per 100 ml but clear serums with no remarkable elevation in triglycerides have been observed in recent years. Five years earlier Gaskins [79] had found the mother to have a triglyceride concentration of 1,600 mg per 100 ml. Neither parent has xanthomas or hepatosplenomegaly and there is no traceable consanguinity in the marriage. The unaffected siblings have completely normal blood lipid levels. Havel [124] found that only the 3 hyperlipemic members had an abnormal response to heparin. Lack of reproducible abnormality in either parent suggests that this is not a simple dominant trait and that it could be a completely penetrant recessive trait in this instance. It is of interest in regard to gene locus that only the hyperlipemic siblings all have sickle cell trait.

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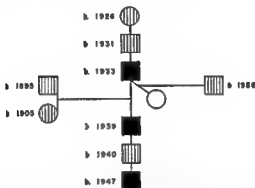


Fig 16-5 Pedigree of the P family representing a variant of essential familial hyperlipemia. The three hyperlipemic siblings have deficient lipoprotein lipase activity in plasma after heparin administration. Elevated triglycerides were obtained in the mother (born 1905) on one occasion [73] but not 5 years later. She is classified here as normal.

The syndrome has appeared in both the white and Negro races and there is no demonstrated preponderance in any ethnic group.

#### *Definition of the Hyperlipemic Trait*

There is need for both more uniformity and more ingenuity in defining the trait or traits responsible for essential hyperlipemia. The minimum study of such patients should include (1) chemical determination of at least plasma cholesterol, phospholipids and glycerides with utilization of UFA and lipoprotein analyses as well as further methods of lipid fractionation if available; (2) determination of plasma lipoprotein lipase activity a few minutes after injection of 10 mg sodium heparin intravenously as determined by UFA production or glyceride disappearance in addition to optical clearing; (3) a glucose tolerance test after adequate preparation by carbohydrate feeding; (4) thorough screening of relatives by the same tests, especially complete plasma lipid analyses; (5) response of the plasma lipids both to reduction to ideal weight if the patient is obese and to diets containing variable amounts of fat.

Upon this base more specific methods of trait definition can be developed and the way opened to understanding of this most intriguing phenomenon.



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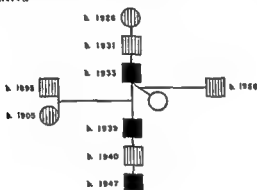


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Upon this base more specific methods of trait definition can be developed and the way opened to understanding of this most intriguing phenomenon.

## COURSE

As defined here essential familial hyperlipemia must be considered a relatively benign condition. Deaths have been reported in three cases. One of the three [96, 130] was almost certainly due to incidental causes. Two of the three brothers mentioned by Harlof [77] died rather suddenly in their youth without a known cause. In the forty some cases reviewed clinical evidence of atherosclerosis has not been present. This factor however entered into the exclusion from this series of the "mixed cases" [90] or possible hyperlipemic variants of essential familial hypercholesterolemia. In these latter cases accelerated development of atherosclerosis is an outstanding complication, this must be considered potentially true for all cases of essential hyperlipemia. Postprandial hyperlipemia has been shown to be associated with development of electrocardiographic signs of myocardial insufficiency [146] decreased coronary blood flow [147] and inhibition of fibrinolysis [148] as well as with acceleration of blood clotting [149]. For these reasons as well as the desirability of avoiding abdominal pain, pancreatitis and eruptive xanthomas and foam cell development in various organs effort should be made to decrease the constant hyperlipemia.

## TREATMENT

The restriction of total fat in the diet to the lowest practical and palatable minimum (30 to 50 gm per day) is the most effective therapy for essential familial hyperlipemia at present. In some cases of both essential hyperlipemia and the hyperlipemia associated with essential hypercholesterolemia accompanied by obesity reduction to ideal weight may produce a dramatic decrease in plasma glycerides. Spaced fat feeding has also been recommended to decrease hyperlipemia [152].

There is evidence that diets extremely low in fat and necessarily high in carbohydrate may cause the low density lipoproteins of high flotation rate and consequently the glyceride concentration to rise [150, 151]. This does not appear to be true for some essential hyperlipemic patients maintained on 5 gm fat diets for short periods (Table 16.5) but the long term effect of such extreme diets has not been explored. It is also not known at present whether different kinds of dietary fat i.e. saturated or unsaturated fats have different effects on triglyceride levels in essential familial hyperlipemia.

Heparin has been used in therapeutic trials by continuous intravenous infusion or repeated subcutaneous injection [103]. All the patients so treated by Lever, Herbst and Hurley [163] responded with some clearing of the hyperlipemia. In none were glycerides reduced to normal levels and there were levels of lipemia below which heparin seemed to be less effective. Most significantly no long standing remission was induced by a

single course of heparin. To be beneficial, heparin would no doubt have to be used as a chronic form of therapy. For this purpose parenteral heparin is quite unsatisfactory, even apart from its potential hazard as an ant clotting agent.

### SUMMARY

1. Essential familial hyperlipidemia is a syndrome characterized by hyperglyceridemia and elevated concentrations of chylomicrons and low density lipoproteins in the blood. The hyperlipidemia is directly related to the intake of fat in the diet. It is often accompanied by eruptive xanthomas, hepatosplenomegaly, and abdominal pain. Rarely there may be associated febrile crises or pancreatitis.

2. The familial occurrence of the syndrome does not of itself define a distinct trait, and more than one biochemical lesion may no doubt give rise to similar clinical features. Somewhat arbitrary grounds have been used here to distinguish the syndrome from other types of hyperlipidemia, such as that associated with essential familial hypercholesterolemia.

3. The cause (or causes) is unknown but appears to be related mainly to defective removal of chylomicrons from the plasma. Defective metabolism of endogenous glyceride may also be involved. One possibly unique pedigree has been found, the hyperlipemic members of which appear deficient in lipoprotein lipase activity. This enzyme may have wide significance in triglyceride and fatty acid metabolism.

4. Males appear to be predominantly affected, but the lack of a clearly definable trait and scarcity of data do not permit proposal of any genetic model.

## ESSENTIAL FAMILIAL HYPERCHOLESTEROLEMIA

### HISTORICAL ASPECTS

The concept of an inheritable tendency toward development of hypercholesterolemia frequently associated with xanthomatosis and atherosclerosis has evolved slowly from the first pertinent observations made over 100 years ago.

Cutaneous tendinous xanthomas were first reported in 1836 by Rayer [154] and in 1851 by Addison and Gull [155]. Occurrence of these lesions in siblings was soon observed by a number of clinicians [156-159], and at the same time it became obvious that they might be accompanied by severe xanthomatous involvement of the heart and great vessels [158, 160-162]. In 1910 Arning [163] reported xanthomatosis in a mother and four of her nine children, at least one of whom died suddenly in childhood. By 1930 the syndrome of familial xanthoma [159] or hereditary xanthoma tuberosum multiplex was well established; more cases than can be accurately compiled have been subsequently observed.

Quinquand (1873) has been credited [164] with first suggesting that

xanthoma development was related to the blood lipids. The studies of xanthoma cells by Pick and Pinkus beginning in 1908 [165] provided the first support for such an hypothesis. Chauffard and LaRoche [164] observed as early as 1910 that hypercholesterolemia accompanied xanthomas in patients with obstructive liver disease. They concluded that "what the tophus is to gout the xanthoma is to hypercholesterolemia." Polltzer and Wile [166] in 1912 expressed a similar opinion after histologic studies of the serial development of xanthoma tuberosum. It was not until 1920, however, that hypercholesterolemia was actually demonstrated in a patient with xanthomatosis without obstructive liver disease [167].

Separation of essential hypercholesterolemia as a syndrome different from other diseases sometimes associated with xanthomas has evolved gradually as classification of xanthomatoses has improved [168-171]. The contributions of Thannhauser and Magendantz [171] and Thannhauser [21] have been especially helpful in this regard. Thannhauser prefers the term *familial hypercholesterolemic xanthomatosis*, separating as a *forme fruste* those patients with essential hypercholesterolemia without xanthomatosis [21]. This terminology is somewhat distracting since it is apparent that xanthomatosis is a secondary manifestation dependent upon duration of hypercholesterolemia [172] and possibly genotype [173].

For this reason the name *essential familial hypercholesterolemia* suggested by Wilkinson, Hand and Fliegelman [173] seems more appropriate. These authors published the first large-scale studies of involved families and numerous similar studies have been subsequently reported (see below). As a result of these the genetics of hypercholesterolemia are partially understood and the clinical manifestations well known.

The frequency with which hypercholesterolemia and atherosclerosis are encountered today however tends to blur the image of essential familial hypercholesterolemia and to complicate the separation of inherited and acquired factors producing hyperlipidemia. As a substitute for cholesterol measurements lipoprotein analyses have been employed by a number of workers especially Gofman and his colleagues [1]. Significant correlations between concentrations of low density lipoprotein classes and atherosclerosis [174] or various xanthomas [175] have been demonstrated. The separation of specific defects in lipoprotein transport as inheritable traits has not yet been accomplished and the biochemical bases for either the hyperlipidemia or hyperlipoproteinemia remain obscure.

#### CLINICAL ASPECTS

##### *Definition and Manifestations*

Essential familial hypercholesterolemia is a syndrome characterized by abnormally high concentrations of cholesterol and phospholipid and

sometimes of glycerides in the blood. Apparently depending upon the severity and duration [172] of the hyperlipidemia and also upon the nature of the abnormal lipid and lipoprotein pattern [175] this syndrome is often accompanied by cutaneous and tendon xanthomas (Figs 16-6 to 16-9) corneal arcus and most seriously a tendency toward accelerated development of atherosclerosis. The syndrome is definitely familial and may involve either sex. It may manifest itself in the first years of life with severe xanthomatous involvement of the skin, endocardium, heart valves, coronary arteries and other organs, ending in sudden death from



Fig. 16-6. Tendon xanthomas on the fingers.

myocardial infarction in childhood or it may be present as nothing more than a slight elevation in plasma cholesterol level with no limitation of the normal life span.

**Diagnosis.** The diagnosis of essential familial hypercholesterolemia depends upon the demonstration of a cholesterol level which is abnormally high for the age, sex, and population group with which the patient is compared. The establishment of a cutoff point between normal and abnormal is arbitrary and varies with each laboratory. For laboratories using accepted methods well standardized, 300 mg per 100 ml or greater should represent a maximum upper limit of normal for any individual. For many laboratories the upper limit will be less than this.

Variation in the expression of an abnormal gene for hypercholesterolemia (see below) and the possibility of multiple causes of essential hypercholesterolemia often make it necessary to use ancillary evidence to establish the diagnosis of the trait. This includes the presence of xanthomatosis or the development of coronary artery disease at a relatively young age in the patient or in his family. Working with population groups frequently affected with hypercholesterolemia or atherosclerosis, Adlersberg et al. [176] have used the presence of hypercholesterolemia in at least three family members to define the presence of the trait. It is very

unusual to find a patient with xanthomatosis for whom at least a suggestive family history is not obtainable. This may be a resultant of the mode of transmission (see below). Hypercholesterolemia can become manifest early in childhood, but it may not be so until the third decade or later [177].

### *Plasma Lipids*

Hypercholesterolemia is nearly always associated with hyperphospholipidemia. Glyceride concentrations are usually normal or only slightly elevated, but definite hyperlipemia may be present.

Plasma lipids in 20 patients with essential familial hypercholesterolemia representing 15 pedigrees are shown in Table 16-6. These patients all definitely familial represent a selected group in that they have sought medical attention. Hence, the proportion having xanthomas and coronary artery disease is higher than would be obtained in a study of their kindred. The distribution of their blood lipid abnormalities is representative of that seen in clinical practice.

**Hyperlipemia.** Six of these patients (R<sub>1</sub> 1, R<sub>1</sub> 2, N<sub>1</sub> 1, Ja 2, GA, and GR) have hyperlipemic serums. Nevertheless, it would be erroneous to classify them as having essential familial hyperlipemia, as the latter syndrome is defined in the preceding section. Patients with glyceride concentrations over 3,000 to 4,000 mg per 100 ml associated with tuberous xanthomas have been reported [83, 85]. Some of these patients have hepatosplenomegaly, abdominal pain, foam cells in bone marrow and spleen, and cardiovascular disease. Hyperlipemia may be present in some hypercholesterolemic members of a kindred, but not in others (Table 16-6) [83, 84, 86]. Such variable patterns of hyperlipidemia are probably different expressions of the same trait, but complete lipid and lipoprotein analyses on complete families are scarce, and the issue is not settled. Borrie [90] has seen hyperlipemia develop in a patient who previously had only hypercholesterolemia.

**Phospholipids.** Monoaminophosphatides (lecithin and cephalin) and diaminophosphatides (sphingomyelin) have been determined separately in several patients with essential hypercholesterolemia [21], and Thannhauser states that only the monoaminophosphatides are elevated [21]. However, his data show an absolute increase in the diaminophosphatides in a case of this syndrome with associated tuberous xanthoma. In view of the reported preponderance of sphingomyelin in low-density lipoproteins [54, 55] and the report of increased sphingomyelin levels during fasting (which may be accompanied by rise in the lower-density lipoproteins) [178], more information is needed on the phospholipid composition accompanying hyperlipemia.

Schoenheimer [11] measured the dihydrocholesterol content of the sterols in a patient with essential hypercholesterolemia and found an

TABLE 16-6 CLINICAL FEATURES IN 20 CASES OF ESSENTIAL FAMILIAL HYPERCHOLESTEROLEMIA

| Patient | Age | Sex | Fe dom<br>xanthomas | Tuberous<br>xanthomas | Coronary<br>artery<br>dis ase | TC<br>mg/100 ml | II<br>mg/100 ml | TG<br>mg/100 ml | FC/TC | TC/PL |
|---------|-----|-----|---------------------|-----------------------|-------------------------------|-----------------|-----------------|-----------------|-------|-------|
| St-1    | 7   | F   | 0                   | 0                     | 0                             | 408             | 368             | 146             | 0.77  | 1.27  |
| St-2    | 20  | F   | 0                   | 0                     | 0                             | 410             | 348             | 79              | 0.28  | 1.18  |
| R-3     | 53  | F   | 0                   | 0                     | 0                             | 435             | 447             | 0               | 0.6   | 0.97  |
| Ja-2    | 11  | M   | 0                   | 0                     | 0                             | 355             | 284             | 31              | 0.6   | 1.75  |
| TH      | 41  | M   | 0                   | 0                     | 0                             | 338             | 298             | 83              | 0.3   | 1.13  |
| N-7     | 40  | M   | 0                   | 0                     | +                             | 478             | 519             | 137             | 0.74  | 0.82  |
| AL      | 20  | F   | +                   | 0                     | 0                             | 408             | 341             | 144             | 0.35  | 1.20  |
| N-1     | 1   | M   | +                   | 0                     | +                             | 363             | 303             | 246             | 0.24  | 0.91  |
| BA      | 43  | M   | +                   | 0                     | +                             | 417             | 340             | 144             | 0.25  | 1.23  |
| BL      | 30  | M   | +                   | 0                     | +                             | 336             | 289             | 175             | 0.78  | 1.19  |
| BO      | 40  | M   | +                   | 0                     | +                             | 441             | 351             | 80              | 0.78  | 1.26  |
| CO      | 43  | M   | +                   | 0                     | +                             | 370             | 358             | 100             | 0.77  | 1.05  |
| HO      | 46  | F   | +                   | 0                     | +                             | 401             | 364             | 194             | 0.25  | 1.10  |
| LA      | 44  | F   | +                   | 0                     | +                             | 454             | 379             | 144             | 0.27  | 1.0   |
| PI      | 44  | M   | +                   | 0                     | +                             | 509             | 503             | 133             | 0.78  | 1.00  |
| R-1     | 50  | M   | +                   | +                     | +                             | 559             | 520             | 177             | 0.31  | 1.06  |
| R-9     | 50  | F   | +                   | +                     | +                             | 684             | 571             | 141             | 0.31  | 1.19  |
| GA      | 60  | F   | +                   | +                     | +                             | 664             | 554             | 644             | 0.30  | 1.70  |
| CR      | 46  | M   | +                   | +                     | +                             | 307             | 398             | 390             | 0.30  | 0.93  |
| Ja-1    | 19  | M   | +                   | +                     | +                             | 495             | 430             | 446             | 0.79  | 1.15  |
| Mean    | 40  |     |                     |                       |                               | 443             | 407             | 388             | 0.8   | 1.12  |
| Normals |     |     |                     |                       |                               | 240             | 20              | 175             | 0.75  | 0.96  |

NOTE: Patients had definite familial histories otherwise selected at random from cases seen by the author. Patients from same kindred are denoted by number. TC = total cholesterol, PL = phloplids, TG = triglycerides, FC/TC = free/total cholesterol. Upper limits of normal from this laboratory not age adjusted.



absolute increase in the small amount of this sterol which is normally present

**Lipoproteins** The hyperlipoproteinemia that necessarily accompanies hyperlipidemia is illustrated in Fig. 16-2. Here data from studies by Gofman et al. [48] have been schematically presented to emphasize the interesting tendency of different lipoprotein patterns to be accompanied



Fig. 16-7 Tendon and tuberous xanthomas (patient Ja 1 Table 16-6)

by different xanthomatous manifestations. Two groups of patients with essential hypercholesterolemia are presented in Fig. 16-2 for clarity and emphasis. Actually, of course, the lipoprotein changes seen in essential hyperlipidemia represent a continuum. While the rather selective lipoprotein changes observed suggest that the trait does not express itself by mere amplification of the normal blood lipoprotein pattern, it must be kept in mind that hypercholesterolemia induced by a number of environmental manipulations or other diseases may involve the same changes in lipoprotein classes depicted in Fig. 16-2. Only the hyperlipoproteinemia associated with biliary cirrhosis may be considered to represent a truly 'diagnostic' pattern [48].

Accompanying the elevated low density lipoproteins there is significant decrease in high density lipoproteins in many patients [48-179]. It has recently been suggested that this is less likely to be true in younger women with hypercholesterolemia [179].

Variation in all lipid levels in the same patient from week to week is common. This is especially true of patients with accompanying hyperlipemia. However, some patients on isocaloric diets in a hospital environment will maintain an elevated cholesterol concentration varying by not



Fig 16-8 Tuberosus xanthomas on the elbows (patient G4, Table 16-6). These lesions may have an inflammatory base and resemble eruptive xanthomas (Fig 16-3).

more than 10 per cent for many weeks. The hypercholesterolemia tends to increase with the age of the patient, and the cholesterol concentration is affected by diet or drugs in a manner similar to that in normocholesterolemic patients.

### Xanthomatosis

The reported frequency of xanthomatosis in essential familial hypercholesterolemia varies greatly. Correlation of its development with duration of hypercholesterolemia [172] but not with any critical level of hypercholesterolemia has been demonstrated. There is also a poorly understood relationship between the type of xanthoma and the abnormal lipid or lipoprotein pattern.

Comparison of Fig 16-2 and Table 16-6 indicates that the garden variety patient with essential familial hypercholesterolemia has elevated concentrations of primarily S<sub>1</sub>, 0 to 10 and 10 to 20 lipoproteins. From the chemical structure of these lipoproteins (Table 16-2) it is obvious that a

high cholesterol to phospholipid ratio and relatively normal glycerides will be found on chemical analysis. If such patients have xanthomas they will usually be located on the eyelids (xanthelasma) or in tendons. Tendon xanthomas (Figs 16-6, 16-7) include both fascial and periosteal involvement.

Xanthelasma has been reported to be accompanied by hypercholesterolemia in only about 50 per cent of patients. Abnormal lipoprotein concentrations have been observed in a higher percentage [1, 180] the



Fig. 16-9 Tubercous and plane xanthomas on the hands

changes being qualitatively similar to those seen in patients with only tendon xanthomas.

When hyperglyceridemia is present elevated concentrations of lipoproteins of higher  $S_f$  value are present (Fig. 16-2) and tuberous xanthomas (Figs 16-8, 16-9) tend to develop. Tuberous xanthomas rarely occur with normal plasma glyceride concentrations but they may occur with tendon lesions (Fig. 16-7). Whether or not hyperlipemia sufficient to cause eruptive xanthomas occurs in essential hypercholesterolemia is subject to the debate concerning classification of hyperlipemia (see above). The simultaneous occurrence of eruptive tuberous and tendon xanthomas has been observed in a rare instance [83].

**Extracutaneous Manifestations** Atheromatous involvement of the arteries especially the coronary arteries and of the endocardium is a

common development in essential familial hypercholesterolemia making this a potentially lethal disease. This has been apparent from the earliest case reports many of which involved sudden death in children because of myocardial infarction or heart failure associated with deformed and incompetent valves. The incidence of clinically detectable coronary artery disease in affected families has been reported as high as over 40 per cent [80]. Clinical signs of atherosclerosis may be present without xanthomatosis. Isolated cases of aortic stenosis have been attributed to hypercholesterolemic xanthomatosis [181-182]. There is greater propensity to develop clinically evident coronary artery disease than cerebrovascular or peripheral vascular involvement [183].

Corneal arcus is common in this syndrome; an incidence of over 50 per cent has been reported in one series of hypercholesterolemic patients [183]. With the exception of corneal arcus, significant extravascular xanthomatosis is not common in essential hypercholesterolemia [81]. Cutaneotendinous xanthomas associated with symmetrical involvement of the central nervous system have been reported [184-185] but at least one case had no hyperlipidemia and the relationship to essential hypercholesterolemia is not clear.

**Associated Findings.** It has been reported that glucose and insulin tolerance test results tend to be abnormal in essential hypercholesterolemia [182] although this is not a universal experience [186]. Diabetes and other endocrine disturbances do not appear more frequently in affected kindred but the possibility of a subtle relationship of carbohydrate metabolism to this syndrome has not been adequately studied.

An interesting feature is the increased incidence of hyperuricemia in patients with hypercholesterolemia [187-188] the highest levels of uric acid being associated with severe xanthomatosis [188]. No explanation for this association is evident. Although hepatomegaly has been observed more frequently in hypercholesterolemic than normocholesterolemic members of several kindred [186] and both hepatic and splenic enlargement may accompany the hyperlipemic forms of this syndrome, liver function test results are normal. Gallstones and cholecystitis may be more frequent than in normocholesterolemic populations [186].

#### THE BIOCHEMICAL DEFECT IN ESSENTIAL FAMILIAL HYPERCHOLESTEROLEMIA

The cause (or causes) of essential hypercholesterolemia is unknown at present. Basically something is awry in the mechanisms for homeostasis of plasma lipid and lipoprotein concentrations, an area poorly understood in the normal state. A fascinating aspect of the syndrome is the tenacity with which abnormally high plasma concentrations of cholesterol and phospholipids are maintained. If cholesterol concentrations are reduced as they sometimes can be by experimental diets containing large amounts

of unsaturated oils or experimental drugs such as estrogens, sitosterols, nicotinic acid or benzmalecane it is remarkable how rapidly they return to their former abnormal levels whenever the diet or drug is removed.

In many patients the abnormal cholesterol and phospholipid concentrations are quite constantly maintained. This is important from the standpoint of experimental measurements of cholesterol metabolism in this syndrome since in the steady state nothing abnormal may be detected. This has been the experience so far in the few measurements of plasma cholesterol turnover that have been made [189]. In these patients whose plasma lipids are in equilibrium at higher concentrations it seems unlikely that cholesterol synthesis or catabolism is uniquely abnormal since one is apparently balancing the other. It is possible that the biochemical basis of this syndrome lies in an increased attraction or requirement for lipid by the plasma.

Since more is known about cholesterol metabolism than about phospholipid, glyceride or lipoprotein metabolism it is necessary for the present to limit discussion of the possible derangement of metabolism largely to the biochemistry of cholesterol. There are other possible causes of essential hypercholesterolemia of course.

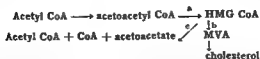
### *Cholesterol Homeostasis*

For a detailed discussion of plasma cholesterol homeostasis with many references the reader is referred to that of Gould and Cook [16]. The liver has been shown to be the organ primarily responsible for the maintenance of plasma cholesterol levels [190-192] and the degree of esterification [193]. Since the free cholesterol of plasma and erythrocytes is in rapid equilibrium [68] it is convenient to consider the blood and liver cholesterol as a double compartment between which an important partition or gradient of cholesterol is maintained. The gut is also intimately involved in cholesterol homeostasis. It has been demonstrated by Hellman and coworkers that cholesterol synthesized endogenously is metabolically indistinguishable [189] from that obtained in the diet.

**Cholesterol Synthesis** The pathway of synthesis of cholesterol is reviewed elsewhere in this volume (Chap. 20). Synthesis of this complex molecule from a simple two-carbon precursor such as acetate involves a number of steps in which the rate of synthesis may be controlled but the exact mechanism is not yet known.

The rate of hepatic cholesterol synthesis from acetate has been shown to be sensitive to a number of factors including the state of nutrition and the amount of cholesterol in the liver [194] which in turn may be a function of that in the diet. These two factors have been especially helpful in recent studies designed to localize a site of regulation [194-197]. Comparison of the effects on rates of incorporation of labeled acetate and mevalonate into cholesterol indicates that the key step is probably

the conversion of  $\beta$  hydroxy  $\beta$  methyl glutaryl CoA (HMG CoA) to mevalonic acid (MVA) Bucher et al [196] have shown that fasting



decreases while factors associated with increased synthesis tend to increase the activity of the phosphopyridine nucleotide-dependent reductase (b) located in the liver microsomes. The activity of the condensing (a) and cleavage (c) enzymes is not affected. It therefore appears that a feedback mechanism for cholesterol homeostasis may have this enzyme locus. Siperstein and Gue t [197] suggest on the basis of *in vitro* studies that cholesterol (or its esters) does not directly mediate this effect.

Many workers have established a relationship between carbohydrate utilization and cholesterol biosynthesis. It has been proposed [198] that the predominant pathway of carbohydrate metabolism might be regulatory. According to this hypothesis, increased utilization of the hexose-monophosphate shunt productive of increased amounts of reduced TPN would lead to increased cholesterol synthesis. Since diabetes is often associated with hypercholesterolemia, a possible relationship of carbohydrate metabolism to essential hypercholesterolemia has been considered. Wilkinson [186] found no abnormality in glucose tolerance test results in a small number of patients with the familial syndrome. Waddell et al [192] who did not prepare their patients with high-carbohydrate diets preceding tests as did Wilkinson, reported abnormal glucose and insulin tolerance test results in a number of patients with essential hypercholesterolemia or hyperlipemia.

Synthesis of cholesterol from acetate has been measured in human beings under several conditions. In hypothyroid individuals there appears to be a decided decrease in the rate of incorporation of acetate. In several patients with essential hypercholesterolemia to whom labeled acetate was given by Hellman et al [189] the rate of acetate incorporation as determined by the appearance of radioactivity in the plasma did not differ from that in several other subjects without hypercholesterolemia. There was also no detectable difference in the curve of decay of labeled cholesterol from the plasma.

These workers also confirmed earlier observations [199] that when labeled cholesterol was fed to patients with some degree of hyperlipemia the ester cholesterol specific activity in plasma was always higher than that of the free cholesterol. Normally there is a short period during which free cholesterol specific activity is higher. This has been interpreted as a possible quantitative metabolic defect in essential hypercholesterolemia.

lemia [199] It is now known however, that the free cholesterol in chylomicrons exchanges or transfers to other plasma lipoproteins much more rapidly than the esterified cholesterol [57] Incoming esterified cholesterol may therefore remain longer in plasma when chylomicrons are more slowly removed altering the usual equilibrium Whatever the mechanism of this phenomenon it has also been observed in the nephrotic syndrome, and it is unlikely to hold the key to the cause of essential hypercholesterolemia

**Cholesterol Catabolism.** Since Bloch, Berg, and Rittenberg first showed that cholesterol was a precursor of the bile acids [200] a number of experiments [103-201] have established that this pathway is the major route for cholesterol catabolism by the liver This conversion involves several steps, not all of them have yet been carried out by cell free systems and the exact biochemical mechanisms are not yet known Their complexity suggests a number of further sites where deficient activity of a single enzyme could alter cholesterol metabolism The terminal three carbons on the side chain must be removed leaving a carboxyl group at carbon 21 and the nucleus must undergo further hydroxylation This must be accompanied by reduction of the 5-6 double bond and epimerization of the 3- $\beta$  hydroxyl group to the  $\alpha$  configuration common to naturally occurring bile acids Bergstrom et al. have demonstrated that the first of these alterations to take place is probably located in the nucleus [103]

The principal end products of these conversions as they are excreted in the bile in man are the taurine and glycine conjugates of cholic and chenodeoxycholic acids [202-203] In the gut the conjugates are hydrolyzed and the acids may be further modified by bacterial enzymes One such modification appears to account for the deoxycholic acid frequently found in human gallbladder bile [203] and possibly the traces of lithocholic acid in bile and feces [204]

Like hepatic cholesterol synthesis, catabolism to bile acids presumably is subject to sensitive control much of which may depend upon the integrity of the excretory and reabsorptive pathways involving the liver and the gut

**Excretion of Cholesterol and Its End Products** Although bile acids are principal end products of cholesterol catabolism in the liver the excretion of both acids and unchanged cholesterol into the gut represents major routes of removing cholesterol from the blood liver compartment It has been estimated that in man 1 to 2 gm cholesterol is excreted into the gut daily from the bile and that an undetermined amount is secreted in other intestinal juices or shed mucosal cells [205] Large fractions of both the sterol and bile acids excreted in bile do not depart in the feces but recycle from the gut to the liver the bile acids via the portal circulation and the sterols via the thoracic duct The amount of recycling of

bile acids is probably considerably greater than the 0.6 to 1 g gm excreted per day in feces [205]. Presumably the enterohepatic recycling of cholesterol is also considerable.

The relative importance of sterol and bile acid excretion as pathways for cholesterol catabolism is not completely known. Calculations of the turnover of cholesterol in the liver plasma pool in man approximate 1.0 g gm per day. This is based on reasonable assumptions of the amount present (12 to 15 g gm) and an estimated turnover time for plasma cholesterol of about 12 days [206]. It is difficult to derive a meaningful turnover rate for plasma cholesterol since it is in exchange with so many compartments at varying rates, but it is interesting that such an estimate of plasma liver cholesterol turnover compares favorably with the turnover of 0.7 g gm bile acids per day estimated for man by Lindstedt [207]. The relative amounts of sterol and bile acids excreted in man have not been firmly established. They appear to vary widely, and more sterol than bile acids may be excreted in certain individuals [208, 209].

It is quite apparent that interference with biliary excretion should affect cholesterol homeostasis, and it has been demonstrated to do so. Biliary obstruction causes hypercholesterolemia and increased hepatic synthesis of cholesterol [210, 211]. Simple diversion of biliary flow from the gut may have the same effect on synthetic rate and is obviated by replacement of bile into the duodenum [205].

The reabsorption of sterols from the gut is related to a number of processes, with no certain assessment of the quantitative importance of each. Some of these factors are degree of esterification in the gut, the amount and kind of fatty acids present, availability of bile, and the formation of chylomicrons. Cholesterol is also converted to other sterols in the gut, apparently through action of bacterial enzymes; the most important of these, coprosterol, is very poorly absorbed. The modification of bile acids by intestinal bacteria theoretically may also affect their enterohepatic cycling.

Preliminary work has suggested that the differences in plasma cholesterol obtained by feeding experimental diets differing in the degree of saturation and chain length of the fatty acid component [150] may be due to effects on reabsorption and excretion of cholesterol and its end products [212, 213]. From the above remarks, it is obvious that there are many ways in which the reabsorption of both sterols and bile acids might be modified and cholesterol homeostasis affected. Plant sterols or sitosterols, which have been thought to compete with cholesterol for esterification in the gut, thus inhibiting absorption, represent one practical application of this theory that has enjoyed some modest success in lowering plasma cholesterol levels. On the negative side, it should be noted that wide variation in cholesterol in human diets has little effect on the plasma concentration. Dietary histories of patients with familial



hypercholesterolemia also reveal no significant difference from normocholesterolemic individuals of the same general population [186]

**The Plasma-Liver-Cholesterol Partition** There are enough examples of widely differing plasma and liver concentrations of cholesterol under both normal and abnormal conditions to indicate that the syndrome of essential hypercholesterolemia might arise primarily from a tendency to maintain an increased plasma liver lipid concentration gradient

Species differences in this gradient normally exist. The rat, rabbit and man for example contain about the same concentration of liver cholesterol (averaging 3 mg per gm wet weight of tissue) while man has a three to fivefold greater concentration in plasma. In the rat biliary obstruction or introduction into the blood of surface-active agents causes tremendous increases in plasma cholesterol and phospholipid concentrations with either no appreciable change [210, 211] or a fall [214] in the liver concentration. A similar phenomenon is present in hypercholesterolemic nephrotic rats [216]

Hepatic cholesterol synthesis is greatly increased after biliary obstruction or administration of a detergent [211, 210] but the primary stimulus may be in an altered equilibrium between liver and plasma. Byers, Friedman and coworkers have suggested that cholate accumulating in the blood in biliary obstruction caused retention of cholesterol in the plasma. They were able to induce hypercholesterolemia in rats with cholate infusion [217]. Using a rather nonspecific method they have reported an increased cholate concentration in essential hypercholesterolemia and other hypercholesterolemic syndromes [218].

Frantz [219] has considered the above problem from an interesting approach assuming that the 'chemical activity' of cholesterol is potentially more important than its absolute concentration. Thus any factor such as wetting agents tending to increase stability of lipid-protein bonds or conditions altering the number of sites available for lipid transport such as an increase in lipoprotein protein or the elaboration of an abnormal protein with peculiar avidity for lipid could disturb the normal plasma liver equilibrium.

**Abnormal Lipoproteins** From available data it cannot be determined whether or not the chemical composition of the various lipoprotein fractions is abnormal in essential hypercholesterolemia. Ultracentrifugal fractions prepared from normal subjects and from patients with essential hypercholesterolemia suggest no distinctive alteration in the ratio of the various lipids present. There is of course some interdependence of chemical composition and density of lipoproteins when the ultracentrifuge is used for isolation of fractions. Lipoproteins of very abnormal lipid composition can be detected however in patients with biliary cirrhosis using existing techniques [220]. Exact comparisons of the chemical nature of protein moieties in lipoproteins from normal and hyperlipidemic

subjects have not been made. The localization of lipoprotein increases in all forms of hyperlipidemia to the group corresponding to low density or  $\beta$  and  $\alpha_2$ -lipoproteins does not offer by itself a specific clue to the cause of essential hypercholesterolemia.

**Phospholipid Metabolism** It has previously been mentioned that hyperphospholipidemia cannot be excluded as a possible cause of hypercholesterolemia. Rarely if ever is one seen without the other. The assumption that phospholipid concentrations rise merely as a part of lipoprotein formation to permit solubilization of excessive cholesterol appears logical enough in hypercholesterolemia induced by feeding sterol to animals but it cannot be extended *a priori* to essential hypercholesterolemia. Hypercholesterolemia has been induced in rats by infusion of either soybean phosphatides or neutral fat emulsions [2,1].

Although it is known that the liver is primarily responsible for synthesis and removal of plasma phospholipids and that various phospholipases present in mammalian tissues promote phospholipid catabolism little else is known about the homeostatic regulation of plasma phospholipid concentrations. The available evidence [38] suggests that neither phospholipid nor cholesterol has an important function in net transport of fatty acids through the plasma and it is unlikely that high plasma concentration of either lipid is a response to a need for increased fatty acid mobilization.

## GENETICS

### *Transmission*

The mode of transmission of the abnormal trait in essential familial hypercholesterolemia has not been satisfactorily resolved despite numerous studies. There is little question that hypercholesterolemia may be transmitted as an autosomal dominant trait. Lack of agreement centers upon the completeness of dominance or whether a double dose of the abnormal gene (homozygosity) leads to higher cholesterol levels developing earlier in life with accompanying severe xanthomatosis and atherosclerosis.

Transmission on the basis of a simple dominant gene was first postulated by Müller [222] on the basis of a few cases and later by Kornerup [223] and Stecher and Hersh [224]. The latter authors compared the incidence of the trait (hypercholesterolemia) as observed by Boas, Parets and Adlersberg [225] in a large group of familial cases with the expected incidence for hereditary characters with complete penetrance. They obtained a 1:1 fit consistent with one parent being heterozygous for a dominant defective allele with nearly complete penetrance and the other parent being homozygous for the recessive normal allele.

In 1948 Wilkinson, Hand and Fliegelman [173] published first hand observations on many members of several kindred and concluded that

transmission was by an incompletely dominant gene, hypercholesterolemia representing the heterozygous or "carrier" state and hypercholesterolemia with xanthomatosis representing the homozygous genotype. Adlersberg, Parets and Boas [68, 225] found their studies of many, smaller kindreds compatible with this interpretation. This was subsequently disputed by Alvord [226], Leonard [227], Piper and Orrild [179] and Harris-Jones [228], who found patients with xanthomatosis who had either offspring or a parent with "normal" cholesterol levels. Similar findings exist in the data of Wheeler [229]. The data of Piper and Orrild [172] were based on a follow up study of the patients of Kornerup [223] and demonstrate clearly that xanthomatosis is also a function of duration of hypercholesterolemia.

Hirschhorn and Wilkinson [230] have recently published findings on three kindreds which support their earlier "incompletely dominant" hypothesis. As a criterion of homozygosity they now use a cholesterol level greater than 400 mg per 100 ml with or without xanthomatosis but they still imply that homozygosity is necessary for development of xanthomas. They explain the failure of other workers [172, 226-227] to confirm their hypothesis as erroneous definition of "normal" cholesterol levels. This criticism appears justifiable for the data of Piper and Orrild [172] and Leonard [227].

It has been pointed out by Epstein, Bloch, Hand and Francis [231] that the crucial mating of two hypercholesterolemic nonxanthomatous persons has not been described a sufficient number of times to determine whether xanthomatosis occurs in 25 per cent of their children. Thus the question of completeness of dominance cannot be answered unequivocally from all the available data. Considering the secondary nature of xanthomatosis and the number of factors likely to affect penetrance of the abnormal gene, the presence or absence of xanthomas may be an unfortunate index to phenotype.

### *Definition of Trait*

It is upon the definition of the presence or absence of the trait (hypercholesterolemia) that all studies of pedigrees depend. It is now known that age [232, 233], diet [150] and probably a multiplicity of other host or environmental factors [234] affect serum cholesterol levels. To these factors are added spontaneous variation in individuals and variation in technical reproducibility of the cholesterol determination.

In the only study of families in which an attempt has been made to define the presence of an abnormal trait by constructing age-adjusted frequency distribution curves of serum cholesterol concentrations, Epstein et al. [231] were able to demonstrate apparent bimodal frequencies in male but not in female members. They critically review the other genetic studies mentioned above and conclude that available

evidence does not unequivocally support any of the available genetic model

A better definition of the normal cholesterol concentration and a clearer separation of inherited and environmental factors affecting blood lipids is needed. It may be necessary to wait for means of biochemical differentiation of the trait before it can be ascertained whether 'hypercholesterolemia' is transmitted by a single gene completely or incompletely dominant or even by multiple alleles

### TREATMENT

At present there is no uniformly effective therapy for maintaining normal blood lipid concentrations in patients with essential familial hypercholesterolemia. A number of medications have been tried which will reduce cholesterol or low density lipoprotein concentrations. Often their effectiveness has been transitory, undesirable side effects have developed or the preparations have been bulky and unpalatable. The only form of therapy that has met the major criterion of usefulness in this syndrome is that it be feasible throughout the life of the patient — dietary modification.

#### *Diet*

The dietary recommendations currently favored are based on both statistical correlations between obesity [235] or dietary fat intake [236] and serum cholesterol levels or coronary artery disease in large population groups and on short term experiments demonstrating the effects of different diets on serum cholesterol and lipoprotein concentrations.

Basically they involve (1) maintenance of an ideal weight, (2) reduction in the intake of fats that are largely saturated or high in short chain fatty acids such as lard, meat, milk, egg and coconut oil, fat, (3) substitution of more unsaturated fats such as corn, cotton seed, peanut, safflower or fish oils. The vegetable oils can be used for cooking and baking.

The response of patients with the familial syndrome to such dietary manipulation under rigidly controlled conditions is shown in Fig. 16-10 and under more practical outpatient conditions in Table 16-7.

It has been advocated [151] that lipoprotein analyses form the basis for determining whether patients should be placed on high levels of unsaturated fat or simple fat restriction depending on the predominant lipoprotein fraction which is elevated. This more sophisticated plan is based on the observation of increases in glyceride concentrations (and very low-density lipoproteins) on a very high-carbohydrate intake.

Skim milk products should be included in these diets as sources of calcium and protein and vitamin supplementation is indicated for initial reducing diets. There is no evidence to support addition of special prepara-

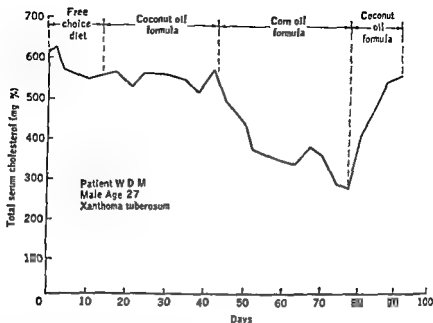


Fig 16-10 Effect of different fats comprising 60 per cent of total calories fed as isocaloric liquid formulas on serum cholesterol in a 27 year-old male with essential hypercholesterolemia. Triglycerides were normal during period of study (Data courtesy of Dr Daniel Steinberg)

TABLE 16-7 COMPARISON OF SERUM CHOLESTEROL LEVELS IN MILLIGRAMS PER 100 ML OBTAINED WITH DIFFERENT DIETS IN OUT PATIENTS WITH ESSENTIAL HYPERCHOLESTEROLEMIA

| Patient | Diet A†  | Diet B†  | Diet C†  |
|---------|----------|----------|----------|
| M L     | 509 ± 32 | 420 ± 23 | 454 ± 24 |
| F T     | 338 ± 3  | 255 ± 18 | 251 ± 8  |
| W B     |          | 353 ± 14 | 368 ± 5  |
| I C     |          | 449 ± 26 | 420 ± 12 |
| C G     |          | 332 ± 16 | 317 ± 16 |

\* Each diet was isocaloric prepared at home according to prearranged menus and was maintained until the cholesterol level was stable for 3 to 4 weeks. Each cholesterol value represents the mean of 3 to 10 determinations.

† Diets: A 50 to 70 gm daily of saturated fat (milk, eggs, meat); B 20 gm of such saturated fat; C 70 gm saturated fat plus 30 to 50 gm corn or safflower oil. Carbohydrate was adjusted to accommodate changes in fat content. Diet C permitted a normal carbohydrate intake. (Study conducted in cooperation with Dr Duncan McCollister.)

tions of vitamins or purified essential fatty acids. The mechanism whereby such dietary changes affect serum cholesterol level is not known but there is no evidence that they are potentially harmful.

### Drugs

Drugs that have been used include both hormones and compounds designed to inhibit cholesterol synthesis, speed its catabolism, or prevent reabsorption from the gut. All estrogenic preparations will cause an increase in high-density lipoproteins and usually some decrease in total cholesterol when administered to hypercholesterolemic patients of either sex. Feminizing doses must be used, and gynecomastia and loss of libido occur in males with distressing frequency. Gastrointestinal distress, water retention, and uterine bleeding also restrict the use of estrogens in females. These effects cannot be selectively avoided by simultaneous administration of androgens or progesterone [237] and no estrogen analogue is yet available which offers dissociation of the lipid and other hormone effects.

Thyroxine or many of its analogues will lower levels of both cholesterol and low density lipoproteins [237, 238] when administered to euthyroid hypercholesterolemic subjects. This frequently cannot be maintained without using doses sufficient to raise basal oxygen consumption, a circumstance not without potential hazard to patients with coronary artery disease [237].

There is no universal agreement about the effects of ACTH and adrenocorticoids on serum lipids, although large doses (100 to 200 mg per day) of cortisone have been found to lower the cholesterol in hypercholesterolemic patients [237]. Other metabolic actions of these hormones make them impractical for long term therapy.

Nicotinic acid in daily doses sufficiently large (3 to 5 gm) to induce vasodilatation and gastrointestinal distress has been reported to lower cholesterol quite significantly in some but not all hypercholesterolemic patients [239]. Buffered salts may alleviate some of the side effects [240].

Sitosterol preparations such as Cytellin (Ili Lilly) in doses of 20 gm per day will produce a small decrease in serum cholesterol in many hypercholesterolemic patients [241]. The required high dosage of this drug and its usual failure to restore cholesterol concentration to normal have limited its acceptance.

A potent inhibitor of cholesterol biosynthesis, MEV 20, has recently become available for treatment of hypercholesterolemia. It apparently blocks the last step in cholesterol synthesis, the reduction of desmosterol (24 dehydrocholesterol) [242]. Since MEV 20 administration is accompanied by accumulation of desmosterol in plasma [242], the therapeutic value of the drug remains to be established.

A number of drugs have had a role in treatment of hypercholes-

terolemia but have been dropped for lack of dramatic success. These include potassium iodide, choline inositol and methionine.

In patients with elevated glycerides and tuberous xanthomas, sodium heparin given subcutaneously in doses of 100 mg per day, or 200 mg three times a week may cause a reduction in hyperlipemia and in the size of the cutaneous xanthomas. Chronic heparin therapy is expensive and not easily adapted to outpatient use. Its use in hyperlipidemia without cardiovascular complications is not recommended at present.

## SUMMARY

1 Essential familial hypercholesterolemia is an inheritable tendency toward development of hyperlipidemia and hyperlipoproteinemia. Depending upon the duration and severity of this manifestation cutaneous-tendinous xanthomas and atherosclerosis may secondarily develop. Death may occur at an early age from the complications of atheromatous involvement of the endocardium or the coronary arteries or there may be no limitation of the normal life expectancy.

2 In addition to hypercholesterolemia and hyperphospholipidemia plasma glycerides may be elevated. The hyperlipidemia is associated with increased concentrations of low density lipoproteins.

3 The disease is presumed to represent an abnormality in the homeostatic mechanisms for regulating plasma cholesterol concentrations. The specific defect is unknown.

4 The hypercholesterolemic trait is apparently inherited as an autosomal dominant. Expression of this trait is influenced by a number of host and environmental factors including age and diet. This makes it difficult to determine precisely the incidence and penetrance of the trait. Two genetic models have been proposed: (1) that the gene is completely dominant; (2) that the gene is incompletely dominant with the homozygous genotype more likely to develop severe hyperlipidemia and xanthomatosis than the heterozygote.

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## Chapter 17

### Infantile Amaurotic Family Idiocy

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*Donald S. Fredrickson*

#### GENERAL ASPECTS OF THE SPHINGOLIPIDOSES

Three diseases characterized by the intracellular accumulation of a different sphingolipid are now recognized. Each is possibly due to an inheritable defect in the metabolism of its typical lipid, but this is unproved. These diseases are known clinically as infantile amaurotic family idiocy (Tay Sachs disease), Niemann Pick disease (Chap. 18), and Gaucher's disease (Chap. 19); they may be differentiated chemically as ganglioside, sphingomyelin, and cerebroside lipidoses, respectively.

Further research may add to this list other familial conditions in which sphingolipids accumulate in tissues. These include Pfäundler-Hurler disease (gargoylism) and certain of the degenerative brain diseases known as the leukodystrophies. Despite the accumulation of gangliosides within the central nervous system, Pfäundler-Hurler disease is still considered to be primarily a disease of mucopolysaccharide metabolism [1, 2]. The leukodystrophies may also be accompanied by lipid accumulation and similar histochemical lesions, but distinctive chemical features have not been uncovered.

The three familial sphingolipidoses involve either nervous or reticulo-endothelial (RE) tissues. The abnormalities in Tay Sachs disease are confined to the nervous system. Niemann Pick and Gaucher's disease may involve only the viscera, or they may be accompanied by extensive brain damage. One of many features of these diseases which remain to be determined is the influence of local tissue factors on expression of the abnormal biochemical trait.

Although chemical analyses have proved that the sphingolipidoses are distinct entities, techniques are still inadequate to distinguish possible subvarieties of each disease. The sporadic detection of less than subtle chemical differences associated with the same clinical and morphologic

findings portends further subclassification and provides a warning against categorical interpretation of some of the older case histories or the most recent theories of pathogenesis.

It is 35 years since the youngest of the sphingolipidoses (Niemann Pick disease) became a recognized clinical entity [3] and fully 60 years have passed since Thudichum disposed of "protagon" as the supposed single chemical constituent of brain and isolated sphingosine and cerebrosides from that tissue [4]. The pace of the search for understanding of the sphingolipidoses has not been rapid, the route passing over the difficult terrain of neurochemistry and through a dark forest of exceedingly complex compounds has been most forbidding. There is now a period of greater expectation. Continuing development of techniques for microchemical analysis of nervous tissue, brain biopsy, chromatography of lipids, and study of enzymes involved in lipid metabolism provide assurance that these diseases may soon be understood at the molecular level.

At present not all the sequential steps in sphingolipid synthesis and breakdown are known and precise information concerning possible enzymatic defects causing these diseases is not available.

## INFANTILE AMAUROTIC FAMILY IDIOCY

### DEFINITION

Amaurotic family idiocy (AFI) is a disease of the central nervous system characterized clinically by blindness, paralysis, and dementia and histologically by abnormal ganglion cells filled with gangliosides and possibly other lipids, demyelination, and proliferation and lipid loading of glial cells.

The symptoms of AFI may begin very early or later in life. When the onset is in infancy the disease is known commonly as Tay-Sachs disease. This form is associated with the characteristic cherry red spot in the retina and is invariably fatal, usually within 1 to 3 years. To describe the later appearing forms, a variety of chronologic or eponymic designations has been employed. A late infantile form (Jansky-Bielschowsky) has been recognized. Lying between this form and the adult (Kufs) variety is a juvenile (Vogt-Spielmeier) form which has been most extensively studied in Scandinavia. Usually becoming manifest at 5 to 10 years of age, the juvenile form differs from the infantile in certain clinical features and especially in racial distribution and genetic pattern [5-6]. Although the ganglioside accumulation in the brain is definitely characteristic for the infantile variety [7-16], the issue is not yet settled for the other forms of AFI [7, 8, 15]. Unless otherwise indicated, further discussion will be restricted to the infantile variety, or Tay-Sachs disease.

## HISTORICAL ASPECTS

In 1881 Tay a British ophthalmologist described a cherry red spot in the fundus of an infant and 3 years later a similar lesion in two siblings [17]. In 1887 Sachs an American neurologist reported clinical and pathologic observations of an infant with blindness and dementia [18]. Within a few years he observed a total of eight cases with the typical retinal change and described the syndrome using the name *amaurotic family idiocy* [19-20]. A large number of cases has been subsequently reported especially from institutions having a high proportion of Jewish patients. Aronson and coworkers have recently summarized clinical and genetic data concerning more than 100 cases [21-21a] and a number of other comprehensive reviews have appeared [22-25].

In 1939-1942 Klenk [7-11] made the important discovery that the brain in infantile AFI contained greatly increased amounts of glycolipids containing a substance later identified as N acetylneuraminic acid or sialic acid (see below). Klenk termed these lipids gangliosides to distinguish them from the cerebroside which also contain sphingosine fatty acid and hexose but not neuraminic acid or its derivatives.

Before it was known that the lipids involved in infantile AFI and Niemann Pick disease were different the similarity of the morphologic changes in the brain and retina in both diseases led some observers notably Pick and Bielschowsky [26] to consider the two conditions as different forms of the same disease. This view is now known to be incorrect. Although these two rare diseases have been reported as possibly occurring in the same sibship [27] it is probable that they have no etiologic relationship beyond chemical similarities in the offending lipids. Some points of similarity between both these conditions and Gaucher's disease (cerebrosideosis) and Pfandler Hurler syndrome (a mucopolysaccharide gangliosidosis) have also already been mentioned.

## CLINICAL FEATURES

*Onset*

Infantile AFI commonly becomes manifest at 4 to 6 months of age in children previously appearing healthy and normal. Typical cases may become apparent much earlier. In one family two siblings had symptoms at birth and died at 18 days and 7 weeks respectively [28-29]. Onset of symptoms may be noted as late as 2½ years of age [30]. No significant correlation has been discovered between occurrence of the disease and order of birth or other prenatal or early postnatal environmental influences. The disease is much more common among Jewish infants than in that of any other parentage.

### *Symptoms and Signs*

AFI often becomes evident insidiously, beginning with listlessness or weakness, retardation in development or difficulties in feeding. The signs progress slowly but relentlessly to further motor deterioration. The earliest sign may not be neurodepressive but neuroirritative such as hyperacusis [21]. In 73 cases studied by Kanof et al [21] the most common initial symptom was hyperacusis.

Visual difficulties may be suspected quite early because of inattentiveness, fixed gaze or other abnormal eye movements. Although the fundus may be normal in the first months of life, the changes in the macula leading to the typical cherry red spot soon become evident. On rare occasions this sign has not been observed, but other "equivalent" changes in the macular area have been present. Optic atrophy is common.

As the neurologic changes increase in severity, epileptiform seizures or convulsions may occur and the child may reach a state of decerebrate rigidity. The average age at onset is about 6 months and the average duration of the disease about 24 months [21a].

The clinical course of the disease has been divided into several arbitrary time phases by Aronson and his coworkers [21, 21a] in order to correlate with certain interesting morphologic changes which are observed. In these children survival is longer than about 15 months. Up to that time the cranial measurements are normal and the brain is slightly subnormal in weight. After this time the brain actually may increase in size and the cranial measurements become concomitantly larger than normal.

### ANATOMIC CHANGES

#### *Nervous System*

Important pathologic changes in AFI are limited to the nervous system. The brain may be abnormally small, normal in size, or large. In patients dying at a very early age, the entire brain is usually atrophic and there is moderate ventricular dilatation [21]. When the onset is later or the rate of progression slower, the cerebral expansion may be considerable with associated marked atrophy of the cerebellum and the brain stem. Regardless of size, the brain is frequently firm or leathery in consistency.

The essential histologic features are as follows: (1) The ganglion cells are enormously swollen or ballooned and distorted. Most of their nuclei are displaced or disintegrated and the cytoplasm fails to show the usual chromophilic Nissl substance. The cytoplasm is sometimes vacuolated and apparently filled with material taking some, but not all, lipid stains. (2) There is demyelination or arrested myelination of the fibers related to the e nuclei. (3) The glial cells proliferate and are converted to foam or fat granule cells. The changes progress sequentially and seem

to increase in severity the longer these patients survive. Far advanced disease is associated with extreme glial hyperplasia, cystic degeneration of the white matter, patchy encephalomalacia and meningeal fibrosis [24]. In this late stage the leptomeninges may be swollen, gelatinous and loaded with foam cells. Thrombi which may be present in cerebral vessels possibly arise from the expulsion of lipid from overloaded endothelial cells into the vessel lumen [24].

The cherry red spot is produced by lesions in the cells in the retinal layer identical to those occurring in the ganglion cells in the brain [31]. The characteristic sign consists of a white zone in the macular region in which the fovea is seen as a small red spot. The grayish macula is thought to be due to edema and swelling of the internuclear layers on either side of the fovea [32] or to swelling and necrosis of the ganglion cells which are most numerous in the region of the macula [22]. Demyelination and degeneration of the optic nerve also occur. The cherry red spot is also seen frequently in Niemann Pick disease. The histologic features of the retinal lesion are practically identical to those in infantile AII. They have been described extensively by Goldstein and Wexler [32].

The ontogenetically most recent areas of the brain are the most susceptible to the disease process [24]. The spinal cord may have similar degenerative changes which are sometimes of such severity that only the axis cylinders remain devoid of their myelin sheaths.

**Histochemical Features.** The abnormal ganglion cells in the brain and spinal cord or outside the central nervous system such as in the sympathetic ganglia or myenteric plexus cannot be distinguished by ordinary hematoxylin-eosin staining from those in Niemann Pick, infantile Gaucher's or I-fundler-Hurler diseases. Even with the use of all available differential stains it may be most difficult to distinguish gangliosides. Diezel [33-34] has reported successful delineation of the lipid in ganglion cells in various neuronal lipidoses. In the AII brain he has found acidic lipid which is PAS positive, sudanophilic and metachromatic in the ganglion and glial cells. Using a modified Bial's test for neuraminic acid he concludes that gangliosides may be demonstrated within these cells in large quantities. Diezel has also reported that some of the acidic glycolipid in the glial and ganglion cells is extracted with difficulty and has interpreted this as strong protein binding of the lipid [34]. Others have expressed caution concerning the interpretation that neuraminic acid in tissues is necessarily present in gangliosides [35]. The staining properties of complex lipids may also differ considerably when they are compared as pure substances and in fixed tissue preparations [36].

#### *Pathologic Changes in Other Tissues*

After Lick and Bielchowsky [26] expressed the opinion that infantile AII and Niemann Pick disease were possibly different manifestations

of the same disease pathologists searched extensively for signs of visceral involvement in AII. There is now universal agreement that the severe involvement of the RE system outside the brain characteristic of Niemann Pick disease does not occur in AII. There are isolated cases however in which lipid accumulation in viscera [37] or hepatic or splenic enlargement has been reported. Diezel [34] reports that in one case of AFI he found small amounts of glycolipid in RE cells of the spleen and liver. No case otherwise typical of AII appears to have been reported however in which ganglioside accumulation outside the central nervous system has been demonstrated by chemical analyses.

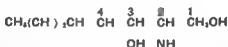
### GANGLIOSIDES

#### Chemistry

Gangliosides contain sphingosine fatty acid hexose hexoamine and sialic acid. The common constituent of the sphingolipids is ceramide the name given to N acyl sphingosine.

**Sphingosine** The long chain dihydroxy amine base sphingosine was discovered by Thudichum in his classical studies of the composition of brain which began in 1874 and culminated in his monograph *Die chemische Konstitution des Gehirns des Menschen und der Tiere* published in 1901.<sup>1</sup> The actual structure of sphingosine was unknown to Thudichum and has only recently been elucidated by a number of studies which have been summarized by Carter [39-40].

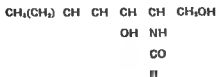
The base as isolated from tissue sphingolipids is mainly D-erythro-1,3-dihydroxy-2-amino-4-trans-octadecene. [40]



The configurational aspects of this molecule may take on special significance as information concerning its biochemistry is unfolded. Erythro refers to the configuration of the substitutions on carbons 2 and 3, trans to the configuration of the 4,5 double bond and D to the configuration of carbon 2. A variable amount of base in naturally occurring sphingolipids is present as the saturated compound dihydro sphingosine [40-41]. Recent gas chromatographic analyses of the periodate oxidation products of sphingosine isolated from human plasma lipids indicate that as much as 10 per cent of the base may be dihydro sphingosine and that there may be other long chain bases of unknown composition similar to sphingosine [42].

<sup>1</sup> The thoroughly annotated biography of Thudichum by Drabkin [38] provides a fascinating description of this scientist and the struggle for recognition of his achievements.

**Ceramide** In the sphingolipids sphingosine is always present as the N acyl derivative in which a long chain fatty acid is attached to the 2 amino group through an acid amide linkage (ceramide)



Ceramide

A ceramide lignoceryl sphingosine has been isolated from extracts of liver lung and spleen [43-45] but there are no quantitative data available concerning either normal or possibly pathologic concentrations of ceramides in tissues

The fatty acid (R) differs among the sphingolipids and in the same class of sphingolipid in different tissues The predominant acids so far demonstrated include palmitic and stearic acid and the  $\text{C}_{23}$  and  $\text{C}_{24}$  acids such as behenic lignoceric nervonic and cerebronic acids

The gangliosides isolated from either normal or AFI brains by Klenk contained largely stearic acid and smaller amounts of lignoceric and nervonic acids On the other hand lignoceric and nervonic predominate in beef spleen gangliosides [10-12 46-48]

The composition of the ceramide fatty acids has often been established solely by melting point determinations Some of the results must be considered tentative and information concerning possible minor components of most of the fatty acids is insufficient The application of gas chromatography to this problem will increase our knowledge considerably

The more hydrophilic group attached to the ceramide through the C-1 hydroxyl group provides the distinguishing characteristic of each of the different sphingolipids In sphingomyelin this is phosphorylcholine (Chap 18) in cerebroside a hexose (Chap 19) and in gangliosides the more complex poly saccharide chain containing sialic acid and hexo-amine

The hydrophilic lipid which Klenk found in such large quantities in the infantile AFI brain had two peculiar properties (1) its strong color reaction with Bial's orcinol reagent typical of furfural or its derivatives (2) its tendency to decompose to black humin substances on heating with mineral acid Klenk called this lipid substance X Its characteristics had been observed earlier by others [39-50] in normal brain and other tissues but in much smaller amounts

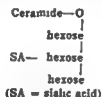
It was soon recognized [9-11] that substance X contained ceramide and hexose but was distinguished from cerebroside by the additional presence of the Bial reactive substance a polyoxyamino acid To the overall glycolipid Klenk gave the name *ganglioside* [11] and to the acid the



name *neuraminic acid* [9] It was determined by Bliz et al [51, 52] and confirmed by Klenk [12] that gangliosides also contained hexosamine

As discussed below, neuraminic acid is now known to be present in gangliosides as *N*-acyl derivatives referred to generically as sialic acid

The structure of gangliosides is still somewhat indefinite In gangliosides of brain and spleen the molar ratio of fatty acid : sialic acid and hexose (including hexosamine) is usually 1 : 1 : 3 [48] Klenk has recently reported that gangliosides from these tissue sources may be mixtures of hexosamine-containing and hexosamine-free compounds [48] A general structural formula for the basic unit as proposed by Klenk [12, 48] is as follows



The third hexose may be replaced by acylated hexosamine The hexose units may be either glucose or galactose Study of the hydrolysis products of certain ganglioside-containing mucolipids (see below) suggests that glucose may be the first hexose unit attached to the ceramide [53, 54]

TABLE 17.1. HEXOSAMINE AND SIALIC ACID COMPOSITION OF BRAIN GANGLIOSIDES (GRAMS PER 100 GM DRY WEIGHT)

| Material                           | Hexosamine | <i>N</i> -acetyl<br>sialic acid | <i>N</i> -acetyl<br>sialic acid per<br>hexosamine |
|------------------------------------|------------|---------------------------------|---|
| Infantile brain, age 2 years       |            |                                 |   |
| Cortex cerebri                     | 0.15       | 0.48                            | 3.2:1   |
| White matter                       | 0.04       | 0.10                            |   |
| Senile brains (mean of 20 samples) |            |                                 |   |
| Cortex cerebri                     | 0.13       | 0.51                            | 3.8:1   |
| White matter                       | 0.012      | 0.025                           |   |
| Tay-Sachs brain, age 1½ years      |            |                                 |   |
| Cortex cerebri                     | 1.09       | 2.15                            | 2.0:1   |
| Nucleus caudatus                   | 0.66       | 1.40                            | 2.1:1   |
| White matter                       | 0.39       | 0.14                            | 2.8:1   |

Source: L. Svennerholm [16], reprinted with permission of Blackwell Scientific Publications, Oxford.

Of all the lipids in brain, only gangliosides appear to contain hexosamine [16]. Svennerholm [16] found higher ratios of hexose to hexosamine and sialic acid to hexosamine in gangliosides prepared from senile brains than in those isolated from an infant brain. He also found a low ratio of sialic acid to hexosamine in the gangliosides from a single case of infantile AFI (Table 17.1). This led him to advance the interesting hypothesis that

loss of hexosamine may be a normal step in the degradation of gangliosides and that such a mechanism may be deficient in AFI

### *Macromolecular Structure of Gangliosides*

There is some evidence that gangliosides may be present in brain as macromolecules or polymers of repeating units. This hypothesis gains some support from the isolation of lipids of high molecular weight which contain large amounts of sialic acid, hexose and ceramide.

One of these lipids is strandin which has been extensively studied by Folch Pi and associates [53-55]. A minimal molecular weight of 11 000 to 13 000 and a particle size of greater than 2.0  $\mu$  have been suggested for strandin [53] and it is maintained that it may have a larger amount of sialic acid than pure gangliosides [53]. Some preparations but not all contain peptide material.

Roenberg and Chargaff [56-59] have introduced the term *mucolipid* largely to cover material similar to strandin which they isolate from brain by techniques derived from those of Folch Pi. They have isolated from brain a mucolipid suggestive of a highly polymerized ganglioside. Analysis of this material obtained from beef brain indicated a molecular weight of about 180 000. For each mucolipid molecule there were about 100 molecules of sphingosine and fatty acid, 234 of hexose (galactose to glucose 8:1), 36 of N-acetylgalactosamine and 151 of sialic acid. Amino acids were also present. They have also found certain differences between the mucolipid isolated from normal brain compared with that from infantile AFI brain. The latter has different solubility properties, lacks the peptide material usually found with the normal mucolipid and is more metachromatic [59].

Bogoch [54] has also proposed a structure for gangliosides in brain as polymers of a basic repeating unit. All workers in this area agree, however, that there is insufficient evidence for conclusions as to the natural state of these lipids and that the definition of these compounds is still largely one of technique of isolation.

### *Sialic Acid*

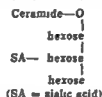
The characteristic acid in gangliosides has recently become a subject of intensive investigation reaching far beyond the area of the sphingolipids. Before Klenk had described neuraminic acid, Blax had isolated a similar compound from bovine submaxillary gland mucin and had given it the name *sialic acid* [60]. Intensive work in both their laboratories and in those of others including Gottschalk in Australia and Yamakawa in Japan resulted in the isolation of these compounds from a variety of tissues and secretions, mostly bound to the carbohydrate portion of mucoproteins.

Although Klenk had originally isolated neuraminic acid from ganglio-

name *neuraminic acid* [9]. It was determined by Blax et al [51, 52] and confirmed by Klenk [12] that gangliosides also contained hexosamine.

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| White matter                       | 0.01       | 0.10                    |   |
| Senile brains (mean of 20 samples) |            |                         |   |
| Cortex cerebri                     | 0.13       | 0.51                    | 3.8:1                                     |
| White matter                       | 0.012      | 0.090                   |   |
| Tay Sachs brain age 1½ years       |            |                         |   |
| Cortex cerebri                     | 1.09       | 2.15                    | 2.0:1                                     |
| Nucleus caudatus                   | 0.66       | 1.40                    | 2.1:1                                     |
| White matter                       | 0.39       | 0.14                    | 2.8:1                                     |

Source: L. Svennerholm [16] reprinted with permission of Blackwell Scientific Publications, Oxford.

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gangliosides [16] it is apparent that both the extraction procedure and the sialic acid method must be as specific as possible. The extraction and determination of ganglioside of brain including the adaptation of column chromatography for this purpose [16] have been critically reviewed by Svennerholm.

A most sensitive and specific color reaction using thiobarbituric acid has been developed recently by Warren [66]. This method will no doubt replace older and less sensitive techniques employing orcinol, resorcinol, diphenylamine, Ehrlich's reagent and other chromogenic substances.

# METABOLISM OF SPHINGOSINE AND GANGLIOSIDES

Parts of the synthetic pathways for ganglioside synthesis may be constructed from interpolations among recently demonstrated enzymatic reactions. These include specifically the synthesis of sphingosine [67-68] of sialic acid [69] and of other glycolipids (cerebrosides). Undoubtedly other reactions involved at some point in the pathway such as the formation of hexosamines are the same as for formation of other carbohydrates. The interesting sequence of steps by which the hydrophilic groups are attached and removed from the ceramide and the possible role of mucoproteins or mucopolysaccharides as donors and receptors of the hexose moieties remain to be elucidated.

## Synthesis of Sphingosine

As partially suggested by *in vivo* experiments of Zabin and Mead [70] and Sprinson and Coulon [71] and recently demonstrated *in vitro* by Brady and coworkers [67-68] the synthesis of sphingosine in brain is accomplished by the condensation of a 16 carbon chain (palmitaldehyde) with a 2 carbon fragment derived from serine. Acetate is a precursor by way of palmitate and formate or glycine by way of serine.

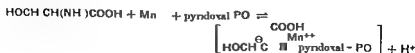
Cell free preparations of brain from immature rats readily incorporate serine and either palmitaldehyde or palmityl CoA into sphingosine. Palmitaldehyde is utilized in the absence of CoA with palmityl CoA. TPNH is required. In this system the following sequence of reactions has been proposed by Brady, Formica and Koval [68].

Palmityl CoA is reduced to palmitaldehyde

O

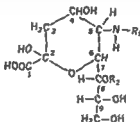


A Schiff base-metal complex is formed with serine, pyridoxal and  $\text{Mn}^{++}$  ions



sides as the methoxy derivative [61] it has become apparent that the compound is naturally present as the  $\Delta$  acetyl,  $\Delta$  glycolyl or  $\Delta$  O-diacetyl derivatives. In human brain gangliosides the sialic acid appears to be mainly the acetyl derivative in beef spleen and horse erythrocytes the glycolyl derivative predominates [48].

It has been proposed [62] that sialic acid be used as the group name for these derivatives and that neuraminic acid be reserved for the basic unsubstituted compound. The structure of neuraminic acid as proposed by Gottschalk [63] and recently established [64] is shown in Fig. 17.1.



| Neuraminic acid |                             | R <sub>1</sub>   | R <sub>2</sub>  |
|-----------------|-----------------------------|--|---|
|                 |                             | H  | H   |
| Sialic acid     | N Acetylneuraminic acid     | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{C}-\text{CH}_3 \end{array}$          | H   |
|                 | N Glycolylneuraminic acid   | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{C}-\text{CH}_2\text{OH} \end{array}$ | H   |
|                 | O O Diacetylneuraminic acid | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{C}-\text{CH}_3 \end{array}$          | $\begin{array}{c} \text{O} \\ \parallel \\ -\text{C}-\text{CH}_3 \end{array}$ |

Fig. 17.1 The structure of neuraminic acid and its naturally occurring derivatives (By permission of L. Warren [64].)

**Distribution of Sialic Acid** Sialic acid is present in the body in mucoproteins in many tissues and the extracellular fluid. As a result of its wide distribution and the demonstration of at least one interesting immunological function for sialic acid, the literature concerning these compounds is considerable. It has been reviewed in part by Gottschalk [63] and by Cornforth et al. [64]. One widely studied reaction is the cleavage of a terminal neuraminic acid unit from the erythrocyte mucoproteins by a neuraminidase present in the cholera vibrio or the influenza virus. Cells so treated are no longer subject to agglutination by the influenza virus. This indicates that sialic acid must function as a prosthetic group for the agglutination.

**Determination of Sialic Acid** The determination of sialic acid in suitable extracts is the present basis for determination of gangliosides. Since the amount of protein bound sialic acid in brain is about 1% that in

that is recoverable in the aqueous phase of a chloroform methanol extract has been studied Radin Martin and Brown [77] found that  $C^{14}$ -galactose was rapidly incorporated into this fraction after a single injection and that the radioactivity subsequently declined with a half life of about 8 days The neutral glycolipids (cerebrosides) were also rapidly labeled and the subsequent decline in radioactivity occurred more slowly than in the ganglioside fraction Mandelstam and Burton [78] observed incorporation of both uniformly labeled D-glucose and D-galactose into gangliosides The rates were age dependent and the maximum incorporation occurred 5 to 10 days postnatally This is much earlier than the time of maximum incorporation of these hexoses into cerebrosides in rat brain [77] The distribution of glucose and galactose in the gangliosides was similar Most was present as galactose or galactosamine relatively little as glucose or neuraminic acid and almost none was found in the ceramide moiety

Judging from the rapid utilization of hexose injected intraperitoneally either glucose or galactose enters the brain from the blood and is incorporated intact into the hexose or hexosamine portions of the gangliosides and cerebrosides The enzymes necessary for the utilization of uridine nucleotides including the UDP galactose 4 epimerase necessary for the interconversion of glucose and galactose are present in brain [79 80]

It is conceivable that the hexoses are not incorporated directly into single ganglioside molecules but first enter a polysaccharide template Ceramide and sialic acid could then be joined to form a ganglioside polymer Since there is no agreement that gangliosides naturally exist in macromolecular form [48] this mechanism is entirely speculative

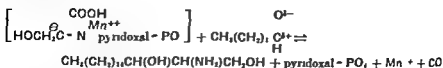
It is of interest that erythrocytes [12 81] and spleen [12] and many other tissues contain glycolipids whose proposed structures appear to lie between those classically representative of gangliosides on the one hand and cerebrosides on the other Klenk has postulated that these lipids may represent transition compounds occurring by alteration in the hydrophilic group attached to the basic ceramide [12] These possible interrelationships between gangliosides and the other sphingolipids may take on more significance when the biochemistry of the developing brain is considered

#### LIPIDS AND THE DEVELOPING NERVOUS SYSTEM

Lipids make up much of the substance of the brain and although their role may be primarily structural they are nevertheless essential to proper function of the neurons The classes of lipids in brain are summarized in Table 17.2 [82]

The brain is far from a finished product at birth and one of the most significant changes during the postnatal development is the increase

Condensation then occurs with decarboxylation of the serine-pyridoxal complex



The resulting dihydrosphingosine is oxidized presumably catalyzed by a flavoprotein



**Ceramide Synthesis** The conversion of sphingosine to ceramide may be the next step in sphingolipid synthesis and presumably involves a reaction between sphingosine and a long-chain fatty acid thioester of coenzyme A [72] Zabin [73] has reported incorporation of serine and palmityl CoA into ceramide by a rat brain homogenate. That ceramides are possible common intermediates in the synthesis of sphingolipids was first postulated a number of years ago by Thannhauser and coworkers [43-74]. It has been recently demonstrated that ceramide is an intermediate in sphingomyelin synthesis [72-75] (Chap. 18).

**Sialic Acid** The N-acetylneuraminic acid present in gangliosides is probably synthesized from pyruvate and N-acetylhexosamine although the reaction has not yet been demonstrated in brain. Comb and Roseman [69] have purified an enzyme from *Clostridium perfringens* which cleaves pyruvate from human serum sialic acid, leaving N-acetyl-D-mannosamine. The reaction is reversible and under the available conditions the enzyme does not catalyze incorporation of either acetylglucosamine or acetylgalactosamine into sialic acid. This interesting finding of mannosamine in human sialic acid is not necessarily at variance with the previously mentioned synthesis of a compound identical to naturally occurring sialic acid from pyruvate and N-acetyl-D-glucosamine [64]. The synthetic conditions used by Cornforth, Firth, and Gottschalk [64] would favor some epimerization of glucosamine to mannosamine. It is postulated that the synthesis of sialic acid from pyruvate (or more readily oxalacetate) and hexosamine proceeds by an intramolecular aldol condensation [64].

**Elaboration of Ganglioside** The further steps by which ceramide hexose, hexosamine, and sialic acid are converted to ganglioside remain to be determined. It has been demonstrated that uridine diphosphate galactose is the primary intermediate in the synthesis of brain cerebroside [76] (Chap. 19); this may very well be true for ganglioside synthesis as well.

The *in vivo* incorporation of labeled hexoses into rat brain gangliosides represented by the nondialyzable and sialic acid-containing glycolipid

In the ganglion cell gangliosides appear in both the nucleus and cytoplasm [88]

The typical lipids of the white matter are cholesterol sphingomyelin and cerebroside [84 85 89 90] and possibly phosphatidyl serine [87] In the period of active myelination of the brain (10 to 40 days of age in the rat [91] and at least 1 to 2 years in the human being [92]) the total brain content of these lipids increases markedly [84 89 90] The lipid in myelin is laminated with protein about the axons of peripheral nerves by the enveloping Schwann cell membrane [93] In the brain there are no Schwann cells and it has been presumed without proof that the oligodendroglia assume the function of myelination [94]

The change in lipid content of the human brain with age is illustrated in Tables 17 1 [16] and 17 3 [15] Although cerebroside has been reported in gray matter (Table 17 3) the gangliosides which also contain hexose must be completely removed from the ' cerebroside fraction' to avoid falsely high values for the latter Folch *et al* for example found in mice that the white matter contributed practically all the cerebroside in the brain [86] Robins *et al* [87] also found very low concentrations of cerebroside in either layer of the monkey cerebellar cortex

Cerebroside should therefore be considered as 'typical' of the myelin sheath and ganglioside is of the ganglion cell In keeping with these findings is the difference in age at which maximal incorporation of hexose into these two lipids takes place In rat brain the maximum synthesis of gangliosides occurs between 6 and 10 days [78] and of cerebroside between 10 and 20 days [76] Klenk [12] also considers sphingomyelin an almost exclusive constituent of the myelin sheath and has made the interesting speculation that this sphingomyelin may normally originate from gangliosides localized in ganglion cells If it can be proved a disturbance of such interconversion would be compatible with the changes found in AFI

#### LIPID CONTENT OF THE BRAIN IN AMAUROTIC FAMILY IDIOCY

**Gangliosides** In 1939 Klenk reported his historic finding that the neuraminic acid content of gray matter was greatly increased (at least ten to twenty times normal) in infantile AFI [7 8] This finding was confirmed by him in several subsequent cases [12 95] in which it was also observed that the neuraminic acid (ganglioside) content of the cerebellar gray and of the dentate nucleus was quite high Gangliosides were also detectable in appreciable quantities in the white matter In his original report Klenk also included analyses from five cases of juvenile AFI in which the total brain content of gangliosides was not increased He speculated that these findings may have been due to more diffuse disease in these cases Other workers have confirmed the ganglioside increase in infantile cases (Tables 17 1 and 17 3) and Cumings



in total lipid content especially notable in the content of certain lipids. Some of the earlier studies of changes in lipids during development have been summarized by Page [83]. Among the most complete of recent studies are those of Brante in rats and human beings [84], Edgar in rabbits [84a] and of Folch Pí [85] and Uzman and Rumley [85a] in the mouse.

TABLE 17.2 QUANTITATIVE SUMMARY OF LIPIDS IN BRAIN (GRAMS PER 100 GM FRESH TISSUE)

| Lipids   | Whole brain | Gray matter | White matter |
|--|-------------|-------------|--------------|
| Total lipids   | 10.4        | 5.0-6.2     | 16.0-20.0    |
| Total phospholipids  |             | 3.1-4.6     | 6.2-9.3      |
| Phosphatidylcholines   |             | 0.6-1.5     | 0.9-1.9      |
| Phosphatidylserines (calc. as stearyl-oleyl)                         | 1.1-1.7     | 0.6         | 1.4          |
| Phosphatidylethanolamines (calc. as stearyl-oleyl)                   | 2.3-2.6     |             |              |
| Sphingolipids (sphingomyelin + cephalin B)                           |             | 0.3-0.9     | 1.8-4.3      |
| Glycosphingolipids (cerebrosides)                                    |             | 0.3-1.9     | 4.1-7.4      |
| Aldehydes in acetal phosphatides (as equimolar palmital and stearal) | 0.35        | 0.14        | 0.63-0.75    |
| Inositol phosphatides (inositol $\times 4.7$ )                       | 0.19        |             |              |
| Strandin and gangliosides  |             | 0.6-0.7     | 0.00-0.01    |
| Sulfatides   |             |             | 0.9-1.2      |
| Phosphatidopeptides  |             |             | 0.1          |
| Total nonsaponifiable material                                       |             | 1.9-2.3     | 6.0-8.3      |
| Cholesterol  |             | 0.6-1.4     | 6.5-8.4      |
| Hydrocarbons   | 0.01        |             |              |

SOURCE: F. N. LeBaron et al. [8] reprinted with permission of The American Physiological Society.

In the brain the neurons have reached their maximum number before birth [86] and the major increase in mass of the brain postnatally is in the white matter accompanying the myelination of the axons emanating from the ganglion cells. Although other lipids, especially phosphatides and cholesterol, are present at birth in the gray matter, the gangliosides are the typical lipid of the ganglion cells. They are present in distinctly higher concentration in the cerebral cortex and basal ganglia than in white matter.

No gangliosides were detected in normal white matter by Klenk [12]. Svennerholm [16] considers gangliosides to be normally present in normal white matter but in much lower concentration than in the cortex (Table 17.1). Using microanalytic techniques, Robins, Eydé, and Smith found that in the monkey cerebellar cortex ganglioside was mainly restricted to the granular layer, much less appearing in the molecular layer [87].

ever to expect the AFI brain to show changes in lipid content characteristic of demyelination [84-96] including a decrease in cerebroside and sphingomyelin.

One unusual case morphologically resembling infantile AFI has been reported by Favarger and Wildi [97] in which both gangliosides and cerebroside were increased in the brain.

#### *Increased Gangliosides in Other Disease*

The accumulation of gangliosides in ganglion cells of the brain is not limited to AFI.

An outstanding example is Pfaundler Hurler disease or gargoylism. This is a familial disease associated with mental deficiency, multiple and varying deformities of bone and cartilage, hepatosplenomegaly and corneal opacities [1-98]. The morphologic changes in the brain are very similar to those in AFI and the ganglioside content is increased [1-2]. In addition, there are relatively large amounts of ganglioside-like lipids and free mucopolysaccharide with a high hexosamine content in the brain, meninges, liver, spleen, lymph nodes and other organs [1]. There may be increased mucopolysaccharide in serum [1]. Dorfman and Lorincz have reported an abnormally high urinary excretion of at least two acid mucopolysaccharides, one of which has been identified as  $\beta$  heparin [99]. It is generally held that this disease is more related to a broad defect in mucopolysaccharide metabolism than to any specific defect in ganglioside metabolism [1-99] although an opposing view has been taken [100].

Increase in brain gangliosides (or sialic acid) has been reported in other diseases associated with demyelination. These include diffuse sclerosis and multiple sclerosis [14] and Niemann-Pick disease (Chap. 18).

### LABORATORY FINDINGS

#### *Sialic Acid in Blood and Cerebrospinal Fluid*

Measurements of sialic acid in a variety of body fluids and tissues have been reported and changes in concentration or the normal distribution with proteins described in a number of diseases [63-65, 101-103]. Saifer, Volk, and Aronson [105] postulate that in AFI there may be a shift of the serum sialic acid from the albumin-bound to the globulin-bound form. According to their data, the total sialic acid is not increased. They also report a lower percentage of protein-bound sialic acid in the cerebrospinal fluid in AFI. Their studies do not permit conclusions concerning a specific abnormality in AFI.

No abnormalities in serum proteins have been found [106]. A possible increase in low-density lipoproteins in some cases has been reported.

has reported increases in juvenile and adult cases as well (Table 17.3). Folch P<sub>1</sub> has also reported increased concentrations of strandin in one infantile case of AII [23].

TABLE 17.3 LIPIDS IN BRAIN

| Lipids             | Normal brain   |                   |                 |                   |
|--------------------|----------------|-------------------|-----------------|-------------------|
|                    | Cerebral white |                   | Cerebral cortex |                   |
|                    | Adult          | 1 week-old infant | Adult           | 1 week-old infant |
| Total phospholipid | 6.3            | 2.77              | 3.85            | 7.5               |
| Sphingomyelin      | 2.1            | 0.52              | 0.98            | 0.54              |
| Cholesterol esters | 0.09           | —                 | 0               | 0.07              |
| Cerebroside        | 5.8            | 0.32              | 1.11            | 0.3               |
| Neuraminic acid†   | 0              | 0                 | 160             | 270               |
| Hexosamine†        | 295            | 700               | —               | —                 |
| (Water %)          | 72.9           | 87.7              | 82.2            | 90.4              |

Brain in three cases of amaurotic family idiocy

|                    | Cerebral white |      |      | Cerebral cortex |      |      |
|--------------------|----------------|------|------|-----------------|------|------|
|                    | 1.5            | 4    | 21   | 1.5             | 4    | 23   |
| (Age yr)           |                |      |      |                 |      |      |
| Total phospholipid | 3.6            | 3.9  | 4.6  | 3.0             | 3.12 | 2.08 |
| Sphingomyelin      | 0.36           | 1.25 | 1.57 | 0.45            | 0.87 | 0.76 |
| Cholesterol        | 0              | 0.32 | 0    | 0               | 0.05 | 0    |
| Cerebroside*       | 2.4            | 1.7  | 2.43 | 1.67            | 1.83 | 1.95 |
| Neuraminic acid†   | 40             | 50   | 55   | 370             | 796  | 360  |
| Hexosamine†        | 877            | 422  | 352  | —               | —    | —    |
| (Water %)          | 79.8           | 74.5 | 72.0 | 82.3            | 84.4 | 83.0 |

Results in grams per 100 gm fresh brain

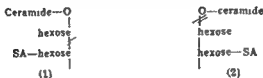
† Results in milligrams per 100 gm dry brain

SOURCE J. N. Cumings [16] reprinted with permission of Blackwell Scientific Publications Oxford

**Other Lipids** There is less certainty concerning changes in content of other lipids in the AII brain. In his early cases Klenk found one case in which the content of glycerophosphatides was low [8]; this was possibly an artifact. In several cases [8] the total sphingomyelin and cerebroside content seems to have been low. This is also true especially for the white matter in the data of Cumings (Table 17.3). Folch P<sub>1</sub> and Lees also report decreases in all other brain lipids in infantile AII [58]. The available data cannot be corrected for the pathologic changes occurring in the total mass of the white or gray matter. It is reasonable how

### Conversion of Gangliosides

Klenk has recently expressed a theory [12] that the lipids typical of myelin i.e. sphingomyelin and cerebroside may arise from gangliosides provided by the axon. Presumably the conversion of ganglioside should occur by one of two mechanisms involving



cleavage of hydrophilic groups from ganglioside to leave either (1) cerebroside or (2) ceramide for synthesis of sphingomyelin. For this type of reaction the ceramide in ganglioside should ideally be similar to that in cerebroside and sphingomyelin. Klenk [12] has observed in brain that the ceramide fatty acid may be predominantly stearic in both gangliosides and sphingomyelin but this is in contrast to the longer-chain more unsaturated acids in cerebroside. The finding by Svennerholm [35] that gangliosides may also contain unsaturated acids in significant quantities leaves this issue unsettled and to be solved by future chromatographic analyses.

The earlier age for maximum incorporation of galactose into gangliosides [78] is consistent with their having a precursor role but more elaborate studies involving specific activity measurements are essential for establishing a precursor-product relationship between gangliosides and the other lipids. It may be argued that synthesis of a less complex compound (cerebroside) via a more complex one (ganglioside) is an awkward route but it is possible for example that ceramide for synthesis of the myelin lipids might be made available to the axon (or glial cell) in the more hydrophilic ganglioside form. The apparent increases in ganglioside content of white matter in ALI and in total brain in other demyelinating diseases are also consistent with a decreased conversion of gangliosides to other sphingolipids.

In the absence of knowledge concerning ganglioside metabolism even speculation concerning the precise nature of the supposed enzymatic defect is limited. The theory of Svennerholm [16] suggesting a normal loss of hexoamine from gangliosides and a possible failure of this mechanism in ALI has been previously mentioned. Little is known about the oligosaccharidases or sialidases which act upon gangliosides. Cleavage of sialic acid or neuraminic acid from mucoproteins by enzymes from microorganisms has been widely studied. Earlier attempts to demonstrate activity against substrate glycolipids from brain or erythrocytes [12, 111]

[107] but there are no comprehensive data supporting abnormal changes in the plasma lipids

### *Enzymes in Extracellular Fluid*

Increased activity of "aldolase" and glutamic-oxalacetic transaminase in serum and spinal fluid and also of "phosphohexose isomerase" in spinal fluid in AFI has been reported by Aronson et al [108]. Many of these same changes were observed in other patients with skeletal muscle atrophy from various causes and were not considered specific for AFI by the authors [108]. It is of interest that serum acid phosphatase does not appear to be elevated in contrast to Gaucher's disease and a rare case of Niemann Pick disease (Chaps 18 and 19).

### *Vacuolated Lymphocytes*

Vacuolization of lymphocytes in peripheral blood has been observed in both AFI and in Niemann Pick disease and there is now considerable literature relative to the possible value of lymphocytes in diagnosis and detection of heterozygous carriers of these traits. Von Bagh and Horting [109] first reported abnormal lymphocytes in full blown cases in Sweden and also found similar vacuolization of lymphocytes in 4 parents of 2 unrelated patients and in 6 of 7 normal siblings of a patient in one family [110]. Further observations of this phenomenon have been reviewed by Spiegel-Adolf et al [106] who also report that similar blood findings occur in infantile cases of AFI. In their studies both parents of 2 patients and a sibling of 1 had vacuolated lymphocytes but parents of several other patients did not. They suggest that the longer the infantile patients survive the more likely vacuolization is to be observed.

The explanation for this phenomenon is not known. It suggests a possible means for detection of heterozygotes however and should be sought in more patients and their relatives until its value has been established. The finding is not specific and care should be taken to eliminate other known causes of such changes in the lymphocytes [106].

### POSSIBLE PATHOGENESIS OF AFI

It is too early to propose a cogent theory of the pathogenesis of AFI. It seems quite likely that the basic defect in this disease is the absence of an enzyme or some other mechanism resulting in the continued accumulation of gangliosides in the ganglion cells. The presence of so much excess lipid causes the cells to enlarge grotesquely and eventually die. As the cells die lipid spills out and is scavenged by the glial cells. Demyelination or a failure of myelination if it has not already occurred takes place either because of the death of the cell or because formation of myelin lipid is impaired by the primary defect in ganglioside degradation [12].

among the Jewish population of the world. The grand parents of their cases came largely from northeastern Europe.

Slome found the consanguinity rates for all racial groups were abnormally high. Roughly 25 per cent of the Jewish parents and 50 per cent of the Gentiles were consanguineous. Of these two groups about half the parental relationships were as close as first cousins.

#### JUVENILE AFI

Although the morphologic changes in infantile and juvenile AFI are similar and the differences in ganglioside increase could be a matter of degree genetic studies suggest that they are not necessarily different expressions of the same mutation. There is no tendency for the infantile and juvenile cases to occur within the same families. The disease has been observed widely in Caucasian peoples notably in Scandinavia and in African and Asiatic races. Jewish cases are extremely rare if they have been reported at all [6]. Sjogren [113] analyzed 59 families in which 115 cases of juvenile AFI occurred and concluded that the Scandinavian strain he was studying was also inherited as an autosomal recessive trait. As with the infantile form the juvenile type involves the sexes equally. Rayner has calculated that the average morbidity in Sweden of juvenile AFI may be 1/40 000 live births. His calculations include the approximation that heterozygotes must occur in that country at a rate of about 1/1 000 [110].

The possible value of vacuolization of lymphocytes in search for heterozygous 'carriers' of the AFI gene or genes has been mentioned above.

The recently published studies of van Bogaert and Klein [27] contain personal observations of 11 families involved with infantile, juvenile or adult cases of amaurotic idiocy. The pedigrees affected with the infantile variety include the interesting family studied successively by Falkenheim and then by R. and L. Rochling in which 13 cases occur in 3 generations. Their pedigrees also indicate that consanguinity is common in affected families. They include an interesting example of double consanguinity within a large Jewish pedigree the disease occurring only in the progeny of these doubly related matings. In non Jewish pedigrees consanguinity was usually present.

A complicated and interesting pedigree presented by van Bogaert and Klein [27] contains the only report of the occurrence of Niemann Pick disease and infantile AFI in the same family. Unfortunately chemical studies were inadequate to establish the diagnosis with certainty or to exclude the possible presence of a new disease with morphologic and clinical features combining those of classical Niemann Pick disease and AFI.

Late or adult forms of amaurotic idiocy appear to be familial and it

were unsuccessful. Rosenberg and Chargaff have recently demonstrated however that sialic acid can be split from normal human brain mucolipid by the *Vibrio cholera* sialidase [59]. It is most interesting that sialic acid was not hydrolyzed from mucolipid isolated from an infantile AFI brain under the same conditions [59].

### *Abnormal Gangliosides*

The highly polymerized ganglioside or mucolipid isolated by Rosenberg and Chargaff from the AFI brain appeared to have some different properties from the normal mucolipid as was described above. They have raised the speculation that the absence of the usual peptide moiety in the AFI mucolipid may have been related to its failure to serve as a substrate for sialidase. Obviously these speculations are of great interest and must await much more research on the purity and physiologic significance of these large lipid aggregates which may be isolated from brain. There is no evidence so far that the chemical components of gangliosides such as the ceramide or hexose moieties, are dissimilar in the normal and AFI brains but the analyses have also not been sufficiently quantitative to prove this conclusively.

Thus the defect responsible for infantile AFI is far from clarified. There is also no certainty that the same defect or trait is responsible for all cases diagnosed as AFI by either the clinician or the analytical chemist. If the defect should be the same presumably in the older children or adults the enzymatic lack (?) is incomplete or partially compensated resulting in less cell death and demyelination.

## GENETICS

### INFANTILE AFI

The existing data support the inheritance of infantile AFI as an autosomal recessive trait. The most comprehensive study is that of Slome [112] who carefully analyzed 135 cases reported up to 1934. The observed frequency of cases in 69 sibships half of which contained four or more members was reasonably close to 25 per cent the portion predicted on the assumption that each parent carried a single recessive gene with expression of the disease only in the homozygous progeny. There was no sexual predilection. Aronson et al [21a] have recently analyzed 122 cases with very similar conclusions. The uniformly semilethal character of the infantile form accounts for the failure to observe direct transmission from one generation to the next.

The disease is much more common in Jews. In Slome's series approximately 20 per cent of cases were in non Jews. In the series of Aronson et al [21a] the percentage was even lower. They also emphasize the indications of an inhomogeneous distribution of the abnormal gene

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has been postulated that the inheritance is recessive [27] but sufficient cases have not been studied to establish the mode with certainty

## SUMMARY

1 Amaurotic family idiocy (AFI) is a sphingolipidosis characterized by dementia paralysis and blindness. The infantile form also known as Tay Sachs disease begins within the first year of life and is fatal by the third. Symptoms may appear later, a juvenile form which usually becomes manifest at age 5 to 10 has also been extensively studied.

2 In all forms of AFI the characteristic pathologic alterations are restricted to the nervous system. In the infantile form it has been conclusively shown that the ganglion cells are overloaded with gangliosides which are hydrophilic lipids containing sphingosine fatty acid hexose hexosamine and sialic acid. The eventual death of the ganglion cells is accompanied by demyelination or failure of myelination and an increase in lipid laden glial cells.

3 The complete sequence of steps in the synthesis of gangliosides is not understood, and little is known of the pathways of degradation or the possible physical state of the gangliosides in brain. It is therefore not possible to determine what enzymatic defects, if any, are responsible for AFI.

4 The infantile and juvenile forms of AFI both appear to be inherited as autosomal recessive traits. The genes have a different racial distribution however and it is not known whether they represent different manifestations of the same basic disease. Vacuolization of lymphocytes in peripheral blood has been observed in some probands and their relatives. It has been suggested that this may provide a means of determining heterozygotes.

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the years 1922-1927 Ludwick Pick correlated the 4 cases into a single entity distinct from Gaucher's disease on anatomic grounds and called the new syndrome "lipoid cell splenomegaly" [5-6]. The disease rapidly became known by the term most familiar today, Niemann-Pick disease.

In the early cases reported as Gaucher's disease Wahl and Richardson [7] and Siegmund [8] found increased lipid phosphorus and cholesterol in the liver and spleen. Further studies by Bloom and Kern [8] and a number of other workers [9-13] established that an increase in phospholipid was indeed characteristic of Niemann-Pick tissues. In 1934-1935 Klenk made the important discovery that the elevated lipid phosphatide in the spleen [14], liver and brain [15] was predominantly sphingomyelin. This was quickly confirmed by others [16-20].

About 100 cases have been reported since Niemann's first case. Videbæk analyzed 73 of these in 1949 and reported for the first time a patient surviving beyond the third year [21]. Crocker and Farber have recently added 18 cases personally observed by them, including others detected in infancy which have had a protracted course [22]. Several cases compatible with Niemann-Pick disease in adults have been diagnosed at autopsy. The first of these were in two Swiss brothers who died at the ages of 29 and 33 [23-24]. In a 51-year-old male studied by Terry et al [25] there were tissue lipid changes comparable to those seen in some infantile patients [22].

As time progresses more cases of Niemann-Pick disease surviving infancy may be observed. An interesting case has been called to the author's attention by Dr. Frank L. Miller [26]. This is a 42-year-old soldier completely asymptomatic who was found by x-ray to have an isolated pulmonary lesion. Biopsy specimens of lung, rib and liver contained histologic and chemical changes compatible with the diagnosis.

A number of reviews of Niemann-Pick disease have appeared. The most recent and comprehensive is that of Crocker and Farber [22]. Others include those of Thannhauser [27], Pfandler [24, 28], Videbæk [21], Baumann et al [29], Canmann [30] and Maurer [31]. The most impressive histologic studies are those made by Bloom [32].

## CLINICOPATHOLOGIC CORRELATIONS

The pathologic changes in Niemann-Pick disease include (1) the accumulation of lipid in reticuloendothelial cells, sometimes everywhere

The author has studied a 19-year-old girl through the courtesy of Drs. Milton Shy and Andrew Engle of the National Institute for Neurological Diseases and Blindness. This patient was found to have splenomegaly at age 4 and progressive neurologic changes beginning at age 17. She is able to live a reasonably normal life at present. In this case a presumptive diagnosis of Niemann-Pick disease has been made by bone marrow biopsy only.

## Chapter 18

### Niemann Pick Disease

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Donald S. Fredrickson

#### DEFINITION

Niemann Pick disease (sphingomyelin lipidosis) is a relatively rare condition characterized by widespread accumulation of lipid mainly sphingomyelin in reticuloendothelial nervous and other tissues. The usual clinical manifestations include hepatosplenomegaly, retarded physical and mental growth, and neurologic disorders which may often include a cherry red spot in the retina. These most commonly develop within the first year of life and progress to a fatal issue by the third year. The disease may occasionally develop later and progress much more slowly. The condition is inheritable but the biochemical basis is presently unknown.

#### HISTORICAL ASPECTS

In 1914 Niemann, a Berlin pediatrician, observed a female infant born of Polish Jewish parents who died at 18 months of a severe malady characterized by feeding problems, abdominal distention, hepatosplenomegaly, facial pigmentation, and edema [1]. At autopsy, striking yellow-white lesions were found in the spleen, liver, abdominal lymph nodes, kidneys, and adrenal glands. The yellow color was due to masses of large cells which stained with Sudan III and which Niemann thought were consistent with the cells previously described in Gaucher's disease. He considered the early age of development and the rapid malignant course in his patient atypical of the available descriptions of the latter disease, however, and chose to report his case as *ein unbekanntes Krankheitsbild*.

Knox, Wahl, and Schmeisser [2] and Siegmund [3] shortly thereafter reported very similar cases but under the diagnosis of Gaucher's disease. Mandlebaum and Downey [4] strongly disputed the diagnosis in one of these cases [2] but they failed to associate it with Niemann's case. During

It is generally accepted from the work of Bloom and of Ilek [6 32] that the NP cells arise from the accumulation of lipid by reticulum cells in lymphoid tissue the resting wandering cells of the connective tissue and perivascular embryonic cells and phagocytes all over the body. The endothelial cells in the liver are also potential foam cells but probably not the endothelial cells in the peripheral blood vessels [32].

Supravital preparations of biopsy material from bone marrow or other tissue often afford the best means of detecting the presence of the NP cells. They are easily distinguished from the Gaucher cells (Fig 19 1) which have a very different and characteristic pattern in the cytoplasm. The morphology of the NP cell is not characteristic of sphingomyelin accumulation however since identically appearing cells may be found in the tissues of patients with severe hyperlipidemia (Chap 16) and have been observed in patients with cephalin lipidosis [33] (see below). There is no absolutely specific histologic stain for sphingomyelin. The Smith Dietrich reaction which is presumed to stain unsaturated fatty acids and hence phospholipids usually stains the NP cells a dark blue or black. A positive reaction is helpful but the degree of staining is variable. Other staining reactions of the cells are described in detail elsewhere [34 35]. A histologic diagnosis should be considered presumptive and corroborated whenever possible with tissue chemical studies.

#### SPLEEN LYMPHOID TISSUE AND BONE MARROW

No doubt because of their high content of reticulum cells these tissues are among the earliest involved in detectable foam (NP) cell accumulation. The spleen increases in size most rapidly between 1 and 2 years of age and may become tenfold enlarged [42]. The splenic pulp may be completely replaced by sheets of foam cells.

The bone marrow likewise contains many foam cells some free others still in the reticular syncytium [32]. Despite such dramatic changes abnormalities in the peripheral blood are not at all remarkable with the exception of vacuolization of agranulocytes. A moderate usually microcytic anemia is common. The leukocyte count varies but not in any characteristic manner. Platelets may occasionally be decreased but the megakaryocytes appear normal. If splenectomy is eventually performed leukocytosis and thrombocytosis promptly occur postoperatively [22].

In nearly all cases careful search will reveal discrete vacuoles in lymphocytes and monocytes in smears of peripheral blood or bone marrow. The cells have an appearance identical to that of the vacuolated leukocyte seen in patients with amaurotic family idiocy. It is reasonable to presume the vacuoles are formed by accumulation of the offending lipid in the diseases. Attempts to demonstrate an increased lipid content of the leukocytes (buffy coat) in blood from patients with Niemann Pick disease have not been successful [22].

Lymphoid tissues including the thymus commonly contain cyto-



in the body and in parenchymal cells of many organs and (2) patchy destruction of ganglion cells and demyelination throughout the nervous system. This pervasive involvement gives rise to a complex but fairly predictable array of symptoms and signs which classically appear in the first 6 months of life and gradually progress.

The first indications of abnormality usually include abdominal enlargement, poor feeding patterns and delays in growth and development. The changes which occur in the usual 2 to 3 year interval between onset and death may perhaps be understood most clearly through a systematic correlation of the anatomic and clinical findings.

#### THE NIEMANN PICK (NP) CELL

The histologic trademark of the disease is the Niemann Pick cell or large reticuloendothelial cell (20 to 90 microns in diameter) shown in Fig. 18-1. The cytoplasm is filled with many droplets or particles which

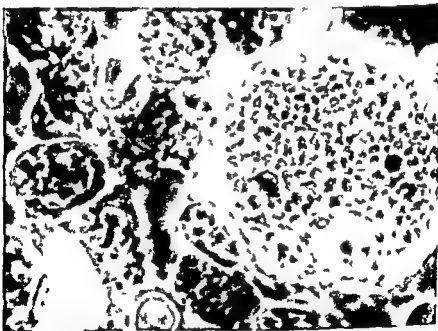


Fig. 18-1. The Niemann Pick cell photographed through the phase microscope in a supravital preparation of bone marrow aspirate. The size of the cell may be compared with that of the adjacent erythrocytes. (Courtesy of Dr. Georg Brecher, National Institutes of Health.)

are usually fairly uniform in size and which give the cell a foamy or mulberry appearance. Occasionally only a few large vacuoles are present, a single nucleus is common but up to 18 have been described [22]. Bloom has discussed the morphology of these cells in most elaborate detail [32].

parietal fissures [37] With rare exception [38] the ventricles are not dilated and the over all weight of the brain is not increased [22] Occasional patients do have prominent frontal bosses and wide fontanelles commonly seen in the older children with amaurotic family idiocy (AII) when extensive gliosis has increased the size of the brain Morphologically the changes are very similar to those described in the latter disease (Chap 17) The ganglion cells are swollen with pale cytoplasm which is often frankly vacuolated There are granular degeneration of the Nissl substance swelling of dendrites and disappearance of the normal fibrillae The white matter is often decreased in volume with a decrease in the normal staining reactions for myelin but without other remarkable microscopic changes [22] Foam cells derived from lipid laden glial cells may appear to replace much of the substance of the brain and are also prominent in the leptomeninges tela choroidea and the connective tissue about the cerebral vessels [6 37 39]

In addition to the brain similar changes occur in the spinal cord the autonomic nuclei and those cells of the adrenal medulla related to the sympathetic nervous system Large vacuoles have been described in the sheaths of myelinated nerves presumably because of accumulation of lipid

Signs of nervous system dysfunction are considered to initiate the late stages of the disease [27] and are usually progressive in development From the degree of anatomic involvement it is understandable that all types of abnormalities in psychic motor and sensory function may occur In the earliest months of life poor feeding habits may be a first sign followed by an obvious lag in mental and motor development Then may follow muscular rigidity involuntary contractions asynergia athetosis tremors and seizures The cranial nerve nuclei are often involved Deafness is quite common because of nerve or central involvement or lesions in the ear itself [29 40]

Mental retardation becomes progressively more severe and has been present in all children before death Previous learning may disappear and the state of the patient may become almost vegetative In some cases developing later in childhood behavior and personality problems may be among the first symptoms [22] Two cases have been reported which are especially notable in that they have remained without detectable nervous system involvement after many years of known disease

The cerebrospinal fluid is normal Nonspecific electroencephalographic abnormalities have been recorded

#### EYES

A cherry red spot has been found in the retina in 20 [22] to 60 [21] per cent of cases This phenomenon consists of a white or grayish macular area in the center of which the fovea appears as a red spot The lesion is similar to that seen in infantile AII although some authors describe

architectural changes similar to those in the spleen. The lymph nodes may be considerably enlarged.

### LIVER

The liver increases in size and undergoes color changes from reddish gray to yellow mainly because of conversion of both Kupffer and endothelial cells to foam cells. The parenchymal cells may also appear vacuolated and may increase in size. It has been suggested that changes in these latter cells may be due not to lipid accumulation but to collection of glycogen or some other degenerative process. Diezel [36] on the other hand has reported that in one case the parenchymal cells were also filled with PAS negative granules which he interpreted as sphingomyelin. The degree of distortion of the liver architecture varies greatly from case to case. There is usually some increase in periportal connective tissue but "cirrhotic" changes are rarely as marked as they may be in Gaucher's disease.

In a few patients jaundice may appear early and persist for a few months [22]. In at least one case [22] evidence has been obtained that this may be because of biliary obstruction produced by large lymph nodes near the hilum. In the absence of jaundice the usual flocculation test results [21, 22] and Bromsulfalein excretion on the rare occasion when it has been measured [22] are normal. Glucose tolerance test results and serum protein concentration are also usually normal even in the most advanced cases.

### THORACIC VISCERA

Histologic changes in the lungs may be difficult to find or they may be spectacular in their extent. On occasion the alveoli have been completely filled with NP cells with prominent involvement of the septums and the area about blood vessels. The lumens of the pulmonary capillaries may contain large numbers of free foam cells. Such changes often produce an x-ray picture of diffuse mottling or miliary nodularity. Occasionally localized infiltration is present. Chronic bronchitis and bronchopneumonia are common and frequently the apparent cause of death.

Foam cells may also appear in the heart interspersed among cardiac muscle fibers. Associated changes in cardiac size or function are uncommon. Edema of the lower extremities is commonly seen late in the disease and may be due mainly to embarrassment of venous return by the enlarged abdominal organs.

### NERVOUS SYSTEM

Throughout the nervous system there occur destructive changes in the ganglion cells, demyelination, proliferation of glial cells and their conversion to foam cells and extensive scarring and fibrosis. The changes may be diffusely distributed [37] or very irregular [22].

The brain is usually hard or leathery with gaping sylvian and inter

compiled from analyses of tissues made by Crocker and Farber from their Case 7 [22] an infant who developed symptoms at 3½ months and died at 21 months. Extensive analyses have also been presented by Thannhauser [27] Terry et al [25] and Klenk [14 15 50-53] a number of studies of individual organs have been made by others [3 7-13 16-20 29 54 55]

In tissues other than brain the analyses may be summarized as showing (1) an increase in the absolute amount of sphingomyelin (often ten to fortyfold) and in the relative contribution of this compound to the total

TABLE 18-1 CHEMICAL ANALYSES (IN GRAMS PER 100 GM FRESH WEIGHT) OF FRESH OR FROZEN TISSUE FROM A CASE OF NIEMANN PICK DISEASE

| Organ        | Lipid          | Cholesterol   | Phospholipid  | Sphingomyelin |
|--------------|----------------|---------------|---------------|---------------|
| Spleen       | 12.6 (2.3-5)   | 2.7 (0.3-0.4) | 7.9 (1.2)     | 4.6 (0-0.9)   |
| Lymph node   | 8.9 (2.3-5)    | 0.6 (0.3-0.4) | 5.1 (1.2)     | 7.0 (0-0.9)   |
| Liver        | 14.8 (3-4)     | 0.8 (0.3-0.4) | 10.1 (1.5-5)  | 6.0 (0.1-0.5) |
| Brain        | 10.5 (2.3-5)   | 0.9 (0.3-0.4) | 1.1 (1.2)     | 6.8 (0.1-0.4) |
| Glycogen     | 6.9 (3.9-5.2)  | 0.9 (0.6-0.7) | 4.3 (3.3-0)   | 1.7 (0.4-0.6) |
| White matter | 8.7 (7.8-14.0) | 1.2 (1.4-3.1) | 4.4 (4.0-6.0) | 1.6 (1.0-1.8) |

B Th r m l A C C k t l (th C 7) [22] d t mb d f m th T 11 III-V d VII  
t t (m th se l b f m B t (r t) h w s th

phospholipid (2) an increase in cholesterol associated in most cases with a rise in free cholesterol and possibly a reduction in the absolute amount of esterified cholesterol [27] and (3) usually smaller but nevertheless definite increases in the non sphingosine-containing phospholipids. The latter have not been extensively fractionated although Chargaff [20] reported that the largest fraction was cephalin in the spleen from one case. In the organs of another case Klenk [15] found no increase in cephalin and only a small increase in lecithin compared with an enormous increase in sphingomyelin. The content of glyceride reported has been variable the determination usually having been made by subtraction of the separately determined lipid fractions from the total lipid. An increase in unaccounted lipid presumably glyceride is also suggested by more recent data [22]. Not all possibility of a new and unexpected lipid component has been eliminated by the available analyses but increased sphingomyelin remains the dominant change.

Klenk originally found the sphingomyelin content of brain increased [15]. In one case Thannhauser et al reported a decrease in sphingomyelin in whole brain although there was an increase in total phospholipid [19]. Some disagreement in total brain lipid content may be more apparent than real since such analyses must reflect a resultant of differing changes in the gray and white matter.

certain differences in size or coloration [41, 42] This sign is not necessarily present at birth and may be unilateral The pathologic changes in the retina in the e two diseases have been described in detail by several authors [43-45]

Additional constitutional abnormalities attributed to dysfunction of appropriate nervous centers [29] include persistent sweating vomiting salivation and fever

### BONES

The conversion of masses of reticular cells in the bone marrow to foam cells might be expected to produce more evident changes in bones Widened medullary cavities occasionally seen at autopsy [30 40 41] are rarely detected by x-ray however and the typical "Erlenmeyer flask deformity" seen in Gaucher's disease has not been reported Osteoporosis no doubt related to nutritional inadequacy and inactivity is common Bone age is usually normal [22] Serum calcium and phosphorus have been frequently measured and found within normal limits

### SKIN

Changes in the skin occur some of which may be related to the infiltration of foam cells in the underlying connective tissue Pigmentation often brownish yellow in color is common It is sometimes diffuse but at other times localized to discrete *café au lait* spots Dark bluish "mongol" spots have also been seen on the skin and oral mucosa [27 48] Suppurative lesions about the face associated with foam cell infiltration and eruptive and infiltrative xanthomas have also been described [22 31]

### OTHER ORGANS

Alterations have been found in every organ So many tissue cell types are loaded with lipid that Iick discouraged the use of the term "lipoid histiocytosis" which Bloom [49] had favored for the disease as too limiting Not all the anatomic changes in epithelial and other cells have expressed themselves in detectable abnormalities in function Thus large yellow adrenal gland variably involved in the medulla and cortex may be present without signs of adrenal insufficiency Other endocrine glands including the gonads thyroid and pituitary and exocrine glands such as the pancreas and salivary glands may likewise be burdened with lipid without evident functional disability Renal function is not grossly abnormal although foam cells are present in the kidney glomeruli and the tubular epithelium may appear abnormal

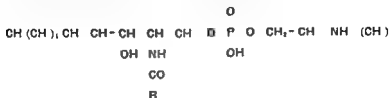
### CHEMICAL ABNORMALITIES

Niemann Pick disease is primarily a sphingomyelin lipidosis, but it is not exclusively so as the analyses in Table 18 I indicate The data are

## SPHINGOMYELIN

**CHEMISTRY**

Sphingomyelin consists of ceramide (N acylsphingosine) to the carbon 1 of which is attached phosphorylcholine [57-59]



The chemistry and biochemistry of sphingosine have been reviewed in Chap 17. The ceramide fatty acids in sphingomyelin characteristically include the 24 carbon acids lignoceric and nervonic but many other acids are undoubtedly present. Klenk [16, 53] and much later Bartsch [55] reported that in normal persons and in Niemann Pick patients the brain sphingomyelin contained mainly stearic acid and that the liver and spleen sphingomyelin contained palmitic, stearic, lignoceric and nervonic acids. Slightly different proportions of the same acids were found in the sphingomyelin of these tissues by Thannhauser and Boncoddio [60]. Chargaff has also analyzed the fatty acids of the sphingomyelin in Niemann Pick spleen [20].

It is important to note however that most of the published analyses have been based on determination of the melting points of the methyl esters of the fatty acids. Gas-phase chromatography will greatly extend the available information concerning these components. Analyses by this technique of the fatty acids present in plasma sphingomyelin in a case of Niemann Pick disease have been made by C. C. Sweeley (unpublished). At least 18 fatty acids were present; the major acid was palmitic but the remainder varied from 12 to 24 carbons in length and included both saturated and unsaturated acids, some of them uncommon and not readily identifiable. Only such complete analyses will ultimately permit determination of whether or not the ceramide in Niemann Pick sphingolipids is normal. From the earlier analyses it has been assumed that it probably is [27-53].

### OCCURRENCE

Sphingomyelin is widely distributed in the body. The highest concentrations are found in the nervous system where this lipid is especially important in the myelin sheath. The lipids of brain are discussed in more detail in Chap. 17. In the myelin sheath of peripheral nerves the sphingomyelin concentration seems to be especially high [61]. In Wallerian de-

Crocker and Larber separately analyzed gray and white matter and found the total lipid content of the white matter less than that of the gray a reversal of the usual composition. This was interpreted as consistent with the deficiency in myelin suggested by the histologic findings. In gray matter the concentrations of lipids were variable all components tending to be moderately elevated or on the high side of normal. That the major change in brain is probably an increase in sphingomyelin has also been confirmed by Cumings [54] and Bartsch [55].

Klenk [52] found a slight increase in the ganglioside content of cerebral and cerebellar gray matter and in one case only in the white matter. In none of the Niemann Pick cases did the increase in gangliosides in any part of the brain approach that found in infantile A1 I. Gangliosides were not detectable in liver or spleen in either normal persons or patients with Niemann Pick disease [52].

#### LIPIDS IN EXTRACELLULAR FLUID

In some cases [11, 21, 29] in which serum lipids have been reported but by no means all [22] there has been a moderate hyperlipidemia. Usually any increase has been in total cholesterol 'neutral fat' or total fatty acids. Occasionally the total phospholipid may be increased [11, 27]. In no case has markedly lactescent serum been reported. In many full blown cases the concentrations of serum lipids and carotenoids may be normal [22, 27]. Most importantly the proportion of sphingomyelin to total serum phospholipids has been demonstrated repeatedly to be normal [27]. The sphingomyelin content of whole blood has also been found to be within normal limits [20].

As with other tissues the lipids of extracellular fluid need to be examined more thoroughly by chromatographic methods. The available analyses of the lipids in ascitic fluid and cerebrospinal fluid are within normal limits.

#### SERUM ACID PHOSPHATASE

In one 3 year old patient Hastrup and Videback [56] found a serum acid phosphatase of 22 Gutman units per 100 ml (normal 2 to 3 units). The pH optimum of this enzyme was the same as that for the enzyme present in the serum of patients with metastatic prostatic carcinoma (pH 5 with phenylphosphate as substrate). The carcinoma enzyme was much more effective in catalyzing hydrolysis of  $\beta$  glycerophosphate.

In a number of other patients with Niemann Pick disease the serum acid phosphatase level has been normal when phenylphosphate at pH 5 has been used as substrate [22]. Thus the finding of a moderate elevation in the concentration of this enzyme appears to be much less common than in Gaucher's disease. The possible relationship of this phenomenon to the etiology of either of these conditions remains to be determined.

is active in catalyzing the esterification of ceramide containing either short or long chain N acyl groups

An interesting feature of the *in vitro* system used by Sribney and Kennedy [65] is the requirement that the sphingosine of active ceramide must have the *trans* configuration of the double bond and the hydroxyl group on carbon 3 must have the *threo* relationship to the amino group on carbon 2. Most of the naturally occurring sphingosine is in the *erythro* form and the implications of this including a possible relationship to Niemann Pick disease are discussed further below.

Studies on the synthesis of phospholipids mainly as determined by the incorporation of labeled phosphorus have been made in a variety of systems. Some of these particularly dealing with the brain have been reviewed by Dawson [66]. Very few studies have been made on the synthesis of sphingomyelin *in vivo* [67]. There are no published experiments comparing synthetic rates in Niemann Pick disease with the normal.

#### CATABOLISM OF SPHINGOMYELIN

Very little is known about the catabolic enzymes which may degrade sphingomyelin and the pathways which the products subsequently follow. There are several points at which the molecule is especially subject to attack. These include the liberation of choline by a cholinesterase followed by release of inorganic phosphate or removal of phosphoryl choline as a unit and the splitting of fatty acid from the ceramide portion. Only the removal of phosphoryl choline by enzymatic means has been demonstrated.

Over 20 years ago Rossi [68] reported the release of inorganic phosphate from sphingomyelin by an extract of spleen. Similar studies using liver and brain extracts were reported by Thannhauser and Reichel [69] and Goebel and Seckfort [70]. Rossi's results have been confirmed more recently by Fujino [71] who showed that splenic extracts were capable of splitting the phosphate-ceramide bond but did not attack the N acyl linkage. This sphingomyelinase activity appears identical to that of certain phospholipase C enzymes which have been isolated from a number of sources.

Phospholipase is the generic term adopted for enzymes acting on lipid phosphatides. Much of the available information concerning them has been summarized by Zeller [72], Hanahan [73] and Hayaishi [74]. The enzymes are commonly named phospholipase A, B, C or D according to their point of attack on lecithin taken as the model substrate [72]. Phospholipases A and B which are esterases attacking the glycerol-fatty acid ester bonds of glycerophosphatides do not have any activity against the peptidic fatty acid linkage in sphingomyelin [72-74].

Phospholipase C isolated from *Clostridium welchii* [75] and *Clostridium hemolyticum* [76] catalyzes the hydrolysis of phosphorylcholine from both



generation (the fragmentation and loss of myelin which follows sectioning of a peripheral nerve) it has been suggested that one of the earliest changes might be the splitting of choline from sphingomyelin, since there is a decrease in the content of ceramide phosphate has been observed [62]

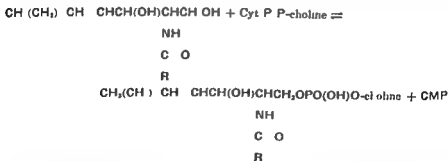
Sphingomyelin comprises roughly 5 to 20 per cent of the total phospholipids in the liver and other viscera. It is also an important constituent of the stroma of blood cellular elements [63]. The absolute and relative concentrations of sphingomyelin have been reported to be higher in (rabbit) leukocytes than erythrocytes [64]. In plasma of man and most species sphingomyelin accounts for 15 to 20 per cent of the total phospholipid. The same proportion holds for other extracellular fluid [27].

Aside from probably important roles in maintaining the structural integrity of the myelin sheath the lipoproteins and other cellular membranes other specific functions for sphingomyelin have not been suggested.

#### BIOCHEMISTRY

The sequential steps through which the synthesis of sphingomyelin normally proceeds in all tissues are not known. Presumably the pathway may proceed from sphingosine to ceramide and finally to sphingomyelin. It has been suggested [65] that at least in nervous tissue gangliosides might be precursors or more specifically ceramide donors for sphingomyelin synthesis. This speculation and the synthesis of sphingosine and ceramide are discussed in more detail in Chap. 17.

Regardless of these uncertainties it has been demonstrated by Sribney and Kennedy [65] that the enzymatic synthesis of sphingomyelin from ceramide and phosphorylcholine does occur in a number of tissues. The addition of 1 choline is accomplished in a manner analogous to the synthesis of lecithin (Fig. 16-4) through the cytidine diphosphate choline pathway.



The enzyme catalyzing this transformation PC ceramide transferase has been found so far in cell free particulate preparations of a number of tissues in several species including liver kidney spleen and brain. The highest activity has been found in chicken liver. The chicken liver en-

is active in catalyzing the esterification of ceramide containing either short or long-chain  $N$  acyl groups

An interesting feature of the *in vitro* system used by Sribney and Kennedy [65] is the requirement that the sphingosine of active ceramide must have the *trans* configuration of the double bond and the hydroxyl group on carbon 3 must have the *threo* relationship to the amino group on carbon 2. Most of the naturally occurring sphingosine is in the *erythro* form and the implications of this including a possible relationship to Niemann Pick disease are discussed further below.

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Phospholipase C isolated from *Clostridium welchii* [75] and *Clostridium hemolyticum* [76] catalyzes the hydrolysis of phosphorylcholine from both

lecithin and sphingomyelin Phospholipase D which splits choline from lecithin has so far been isolated from plant sources only [77] and its activity against sphingomyelin has not been reported It should be noted that a number of authors, following the nomenclature proposed early by Contardi and Ercoli [78] reverse the terminology used above for phospholipase C and D activity

A comparison of phospholipase C activity (or "lecithinase D activity" in the terminology preferred by the authors [22]) in normal and Niemann Pick spleens has recently been made by Crocker and Farber [22] Extracts of spleen were incubated with a lecithin suspension and the release of acid soluble phosphorus determined There was no detectable difference between the activities of the extracts from the normal and abnormal tissues They also found that the sphingomyelin in the Niemann Pick tissues proved to be a readily utilizable substrate for *C. welchii* phospholipase C Experiments similar to the latter had also been reported earlier by Freudenberg [79]

As has been pointed out by Thannhauser [27] and other authors the ceramide released from sphingomyelin is theoretically a pivotal compound and possibly available for utilization for synthesis of other sphingolipids This possible interconvertibility has been discussed in Chap 17 It is extremely difficult to devise experiments *in vitro* with sphingomyelin and related compounds which may duplicate the equilibria and other conditions obtaining in the whole organism The substrates are relatively water insoluble and the activity of certain of the phospholipases has been shown to be influenced greatly by a number of factors including the presence of phospholipids other than the major substrate [80] The elucidation of the pathways with which Niemann Pick disease is intimately concerned is thus a problem of considerable magnitude

## DIAGNOSIS

Niemann Pick disease may be strongly suspected from the combination of hepatosplenomegaly and neurologic signs and symptoms A tentative diagnosis can be made from the additional finding of NP cells in bone marrow aspirates or tissue biopsies The diagnosis should be confirmed by the chemical determination of a pre dominant increase in sphingomyelin in the involved tissues

This point is emphasized particularly because of the interesting report of Baar and Hickmans [33] of a disease in two siblings which might have passed as Niemann Pick disease had analyses of tissues not been made The clinical features were compatible with the latter diagnosis although the NP like cells yielded a peculiar color with the Smith Dietrich reaction The abnormally elevated lipid in the organs proved to be an inosamine-phosphatide The plasma phospholipid partition was also abnormal It is

conceivable that this 'cephalin lipidosi' or another similar disorder has already been erroneously included among the cases of "Niemann Pick disease" which have been reported.

It is also possible of course that not all cases of sphingomyelin lipidosi arise from the same biochemical disorder. In every case specimens of tissues should be carefully stored by refrigeration for future reference. Such tissues are relatively rare and only by examining them with each appropriate new technique for determining lipid or enzyme content will the cause of this distressing syndrome be ascertained.

A rather simple method for determining the sphingomyelin content of tissues useful in laboratories equipped to do lipid phosphorus determinations has been described [22]. It is based on techniques developed by Schmidt and coworkers which are presented more extensively elsewhere [81]. Lea, Rhodes and Stoll first described the successful separation of phospholipids on silicic acid columns [82] and recent papers by Hanahan et al. [83] and Philips [84] are representative of further modifications of this technique made by many laboratories. Marinetti et al. [85] have described useful systems for quantitative paper chromatography of lipid phosphatides.

## TREATMENT

There is no specific treatment at present. Splenectomy, repeated blood transfusions, *p*-aminosalicylic acid, nitrogen mustard, folic acid, antagonists, lipotropic substances, cortisone, antibiotics, liver extract, and massive doses of vitamin A have been tried without remarkable effect on the progress of the disease [22]. Anterior pituitary extract was reported to have reduced the size of the spleen and to have ameliorated some of the symptoms in one case [28]. Radiation may be helpful in local control of infiltrative skin lesions [22].

## ETIOLOGIC POSSIBILITIES

The absence of inflammatory or other detectable signs of a disease to which the accumulation of sphingomyelin and other lipids might be secondary makes it extremely likely that Niemann Pick disease is a primary lipidosi. Two patients with apparently primary carcinoma of the liver associated with widespread occurrence of NP-like cells [22, 86] and increased sphingomyelin content of other organs [22] have been reported. In one [87] severe hyperlipidemia was also observed. The findings are not enlightening so far as the etiology of Niemann Pick disease is concerned except to emphasize that similar tissue changes may have a polyvalent basis.

A defect in sphingomyelin metabolism is most highly suspect as the

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A defect in sphingomyelin metabolism is most highly suspect as the

cause of the primary lipidosis. In assuming that this is the site of the metabolic error, it is necessary also to explain the excessive concentrations of cholesterol and other lipids other than sphingomyelin in most of the tissues examined. It is not possible to do this at present. There are however convenient examples among the various hyperlipidemias (Chap. 16) suggesting that a single lipid is rarely if ever uniquely elevated in the serum. This is presumably because of the requirements imposed by lipoprotein structure, probably similar requirements obtain in tissues as well. This analogy may be carried further of course by recalling suggestions (Chap. 16) that excessive elaboration of a protein with special affinity for lipid might be responsible for certain types of hyperlipidemia. This concept has been advanced by Uzman [87] as being related to the cause of another sphingolipidosis, Gaucher's disease. Search for evidence of abnormal protein binding of lipid in Niemann-Pick tissues has not been successful [22].

It does not seem likely that an increased net transport of sphingomyelin through the extracellular fluid from a single site to all other organs is important. Thannhauser [87] has stressed that normal plasma concentrations of this lipid minimize this possibility. Regardless of whether or not there is increased sphingomyelin transport, it must be secondary to some other abnormality in the production or degradation of lipid. Several experiments have been conducted in which sphingomyelin emulsions have been injected into animals [88-90]. Cells similar to the NP cell were said to have developed in several tissues, but on at least one occasion they were not reproduced [91] and the experiments do not shed much light on pathogenesis. It is surprising indeed that there is no increase in sphingomyelin in extracellular fluid. The reticuloendothelial cells seem to retain to the end a remarkable affinity for the excess lipid.

In consideration of a defect in sphingomyelin metabolism, three or four major possibilities exist. For and against each, some intuitive argument or fragmentary experimental evidence may be raised. The first is that the synthesis of a normal sphingomyelin molecule proceeds unchecked and at a reckless pace which far outstrips dissimulation of the molecule. A possible analogy is gout (Chap. 21) in which both abnormal diversion of substrate to the product uric acid and a faulty control mechanism are suspected.

A second possibility is the synthesis of a structurally abnormal sphingomyelin molecule resistant to the action of a normal complement of degradative enzymes. One possibility of this nature has been raised by Sribney and Kennedy [95] who found that only ceramide containing sphingosine in the *threo* configuration could serve as substrate for their PC-ceramide transferase reaction. All naturally occurring sphingosine appears to be in the *erythro* form [92] and Sribney and Kennedy postulated that isomerization might be a final step in normal sphingomyelin

synthesis. They further called attention to the possibility that such a step could be missing in Niemann Pick tissues and that the resulting *threo* sphingomyelin might be resistant to further catabolism. Neither such a normal isomerase reaction nor the stereoisomerism of the sphingomyelin in Niemann Pick tissues has yet been established. As noted earlier the fatty acid composition of sphingomyelin must also be determined with greater accuracy. One argument against the possibility of an important alteration in sphingomyelin is the apparent availability of this molecule in Niemann Pick tissues as a substrate for phospholipase C hydrolysis [22].

As mentioned above and in Chaps 17 and 19 in ganglioside and cerebroside lipidoses an abnormal macromolecular structure or protein binding of the offending lipid has been suggested as the underlying abnormality. Polydiaminophosphatides were described many years ago in lipid isolates from tissue [68-93] but it is probable that these were artifacts [94] and in the intervening years no further evidence that sphingomyelin might exist in tissues in any type of polymer has been presented.

The final possibility seems the most likely that there exists in Niemann Pick disease some inheritable alteration in or absence of an important catabolic enzyme. Phospholipase C activity is present in these tissues [22] as it is in normal tissue but beyond this nothing is known of the routes or products of sphingomyelin catabolism. Until the normal pathways are established it is impossible to speculate knowingly on possible single enzyme defects that might account for Niemann Pick disease.

The available evidence indicates that whatever the basic defect it expresses itself widely perhaps involving most tissue cells. Presumably the dramatic and early [32] involvement of the reticuloendothelial system indicates that the responsible defect is present in these cells and that they are not merely exercising their well known capacity for lipid phagocytosis. Nothing definite is known of the relative capacity of reticuloendothelial cells to make and degrade sphingomyelin. Of interest are the post mortem findings in a stillborn infant with Niemann Pick disease examined by Burne [95]. The most advanced changes were present in the spleen, thymus, lymph nodes and adrenal cortex. No foam cells were present in the liver but the parenchymal cells were distended with glycogen and Kupffer cells were swollen. The organ was not analyzed chemically. In the kidney no deposition of lipid was detected histologically but it contained abnormally large amounts of sphingomyelin. Autonomic ganglion cells throughout the body were undergoing typical destructive changes and in the brain the ganglion cells of the pons were abnormal. Those sections of the cerebellum and cerebral cortex which were examined appeared normal.

It appears then that lipid begins to accumulate first not only in tissues



rich in reticulum cells but also in the more "primitive" ganglion cells. Such early predilection may be an expression of an advanced functional capacity of these cells. In the brain, sphingomyelin begins to accumulate after birth [62, 96] and major changes in the cerebral cortex would be expected to be somewhat delayed.

## RELATIONSHIP TO TAY SACHS DISEASE

The changes in the brain in Niemann Pick disease afford an especially interesting comparison with those in infantile amaurotic family idiocy (Tay Sachs disease) (Chap. 17). This is especially true with respect to the relationship suggested between gangliosides elevated in Tay Sachs disease and sphingomyelin. It has been considered that in the brain at least gangliosides might be the precursor of sphingomyelin [53], the latter being elaborated mainly by the axon or glial cell responsible for the laying down of myelin. Yet in Niemann Pick disease it is remarkable that the ganglion cells themselves seem to be distended or "ballooned" until they die, resulting in demyelination or failure to myelinate in a manner identical to that in Tay-Sachs disease. The only difference in the brain would seem to be in the lipid accumulated. The establishment of the pathways relating sphingomyelin and gangliosides is awaited with interest in this regard. The occurrence of the two lipidoses in the same pedigree has been reported [97] but the diagnoses were not substantiated by chemical analyses. Until such evidence is obtained there is no firm basis for assuming that the two diseases are very similar biochemically, e.g. involving the same enzyme system with some other determinant directing the nature of the abnormal lipid which accumulates.

## GENETICS

Although Niemann Pick disease is rare, it has been observed too frequently among siblings to be due to chance. In a total of 91 cases [21, 22] at least 12 have involved more than one patient in a sibship. Three siblings have been affected in at least two families [22, 48]. There are in addition a number of families in which one or more siblings of the proband have died following an illness suggestive of but not proved to be Niemann Pick disease. The sex distribution is about equal.

More cases have occurred in Jews than in any other ethnic group—35 of 73 cases in which the race is known in the two largest series [21, 22]. Most of the remaining cases have occurred in other Caucasians. There has been one case in a child of Syrian parentage [23] and one definite another probable case from the mating of an American Indian Negro father and a Portuguese mother [22]. Cases have also been reported from Turkey [98] and Japan [99]. Of special interest are four cases of Crocker and Farber [22], all of French Canadian Catholic ancestry who had a

similar and unusually protracted course. There was no traceable interbreeding but the same family name occurred in several of the pedigrees.

Of 79 matings producing at least one child with Niemann Pick disease [21 22 28 100] six are known to have been consanguineous. It is difficult to be certain of the rate of occurrence in involved families particularly as has been pointed out [22] since parents may tend to limit the size of their families after the appearance of an involved offspring. Crocker and Farber [22] found 17 definite cases in a total of 40 contemporaries among their 13 families with more than one child. No doubt because of its semi-lethal character the disease has not been reported in more than one generation in an affected pedigree. Thus the available evidence indicates that Niemann Pick disease is probably inherited as an autosomal recessive trait [101] but this has not been firmly established.

Some attention has been given to the problem of detecting heterozygous carriers although in many cases no family study at all has been reported. Two pedigrees have been examined by Pfandler [28]. The first is that from the Brevine valley in Switzerland from which came the first two cases reported in adults. These two brothers whose disease was proved at autopsy had 12 siblings. Two of these also had hepatosplenomegaly but were over 50 years of age at the time of examination. Pfandler considered these two siblings as having latent macrosymptoms of the disease. Several other members of this pedigree were found to have an increase in serum total fatty acids. The cholesterol values were normal and the phospholipids not determined. The same type of hyperlipidemia was described in the siblings and a patient of a Danish family in which two definite cases of Niemann Pick disease appeared. These changes in the blood lipids which Pfandler calls "microsymptoms" are interesting but require further evaluation.

In two separate cases both sets of parents were found to have normal bone marrows and serum lipids by Crocker and Farber. In one case [22] the father and two paternal granduncles had splenomegaly. In one of the latter bone marrow and splenic aspirations revealed abnormal cells differing from NP cell but comparable to others which have been previously described [102]. A search for vacuolization of agranulocytes in the peripheral blood in parents and siblings of several cases has not been rewarding [22].

Until a clear-cut trait can be defined it will be difficult not only to determine the precise mode of inheritance of Niemann Pick disease but also to solve the related problem of whether more than one biochemical disorder may be currently considered as a single disease.

## SUMMARY

1. Niemann Pick disease is an inheritable condition in which lipid accumulates in reticuloendothelial and other cell types throughout the

body. Although every organ may be involved, the changes occurring in the nerve ganglion cells are especially destructive, leading to cell death and demyelination.

2 The clinical features include hepatosplenomegaly, retarded physical and mental growth, and severe neurologic disturbances. A cherry red spot in the macula may be present. Symptoms usually develop by 6 months of age, and the disease is usually fatal by the third year. Some cases beginning in infancy may survive much longer. Sporadic cases have been detected in adults.

3 The accumulated lipid in the tissues is mainly sphingomyelin. Other phospholipids and cholesterol may also be present in abnormally high concentration.

4 The disease is probably transmitted as an autosomal recessive trait. It is most common in children of Jewish parentage.

5 The underlying biochemical disorder, most likely a defect in sphingomyelin metabolism, is unknown.

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body. Although every organ may be involved, the changes occurring in the nerve ganglion cells are especially destructive, leading to cell death and demyelination.

2. The clinical features include hepatosplenomegaly, retarded physical and mental growth, and severe neurologic disturbances. A cherry red spot in the macula may be present. Symptoms usually develop by 18 months of age, and the disease is usually fatal by the third year. Some cases beginning in infancy may survive much longer. Sporadic cases have been detected in adults.

3. The accumulated lipid in the tissues is mainly sphingomyelin. Other phospholipids and cholesterol may also be present in abnormally high concentration.

4. The disease is probably transmitted as an autosomal recessive trait. It is most common in children of Jewish parentage.

5. The underlying biochemical disorder, most likely a defect in sphingomyelin metabolism, is unknown.

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## Chapter 19

### Gaucher's Disease

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*Donald S. Fredrickson and Alan F. Hofmann*

Gaucher's disease (cerebroside lipidosis, cerebrosideosis) is a rare familial disease in which characteristic cells loaded with cerebroside are found in reticuloendothelial tissues. It may become clinically apparent at any age. In the adult it progresses slowly and the manifestations are directly related to enlargement and destruction of tissues filled with masses of Gaucher cells. When it appears in infancy the process is more severe and is usually accompanied by crippling lesions in the nervous system which tend to hasten the course to an early death.

This condition was first described by Philippe Gaucher in 1882 [1] and for over a half century the clinical course, relentless and monotonous in its reproducibility, has been well known. During this time significant advances have been made in disclosing the chemical abnormalities, but the basic metabolic lesion in Gaucher's disease is still unknown. This chapter will be devoted more to a review of the relevant biochemical area than to the fine detail of the clinical manifestations. These latter have been carefully reviewed by a number of authors [2-9], the comprehensive review of Thannhauser being the most recent [9].

#### HISTORICAL ASPECTS

In his description of the first case, Gaucher called attention to the chronic progressive course, the splenomegaly, hepatomegaly, and skin pigmentation, and considered that the peculiar cells in the spleen, which were later to bear his name, were evidence of a primary neoplasm of that organ. He later revised this opinion [10], suggesting that the major process was connective tissue proliferation associated with enlargement of parenchymal cells. As more cases were discovered, the concepts of etiology gradually evolved through a theory of toxic origin [11], of diffuse systemic disease of the lymphatic hemopoietic system [10], to the deposi-

tion of a foreign substance in the reticulum cells [12] either carried there by the blood [13] or elaborated *in situ* [9]

Following Marchand's suggestion that the abnormal cells contained a foreign substance [12] a number of investigations indicated that this material was 'lipoid' in nature. These early studies are thoroughly reviewed by Epstein [14] who working with Lorenz [14-15] found the Gaucher spleen to contain large amounts of alcohol soluble acetone-insoluble material. Spurred by their findings Lieb [16-18] in 1924 isolated from this tissue large amounts of the cerebroside kerosin which had been described 50 years earlier in brain by Thudichum [19-20]. Many subsequent analyses have brought confirmation that the typical lesion in Gaucher's disease contains excessive amounts of cerebroside. In most cases however the cerebroside accumulating in the viscera is not the normal galactocerebroside but contains glucose. This was first discovered by Aghion in 1934 [21] and confirmed by more certain analytical techniques by Halliday et al [22] in 1940. The finding of increased acid phosphatase activity in plasma and Gaucher tissues [23, 23a] is the most recent advance of possible major significance.

The occurrence of Gaucher's disease in siblings was noted in very early case reports [11-24-25] and the few hundred case reports since published clearly indicate that the disease has some hereditary basis.

## CLINICOPATHOLOGIC CORRELATIONS

The diagnosis of Gaucher's disease has been made at ages as disparate as 1 week [2] and 79 years [26]. About one third of the reported cases have been detected in infancy or childhood. The chemical abnormalities found in the tissues of so-called infantile, childhood and adult forms have been indistinguishable. It is assumed that the disease represents a continuum with significant differences in the nature of the manifestations depending upon the time of appearance or rate of development.

In infancy the process leading to cerebroside accumulation is most widespread. In addition to the usual enlargement of the spleen and liver marked lymph node involvement is common and Gaucher cells may appear in lung, thymus, adrenal cortex and other tissues [7]. Most importantly there also occur destructive lesions in ganglion cells in the brain and the clinical course may be dominated by signs of gross central nervous system dysfunction. These include poor feeding patterns, laryngospasm, spasticity and a variety of other abnormal signs [2]. In addition there are mental retardation, eventually total idiocy and cachexia and finally death, often from intercurrent infections.

In most children who first develop signs of the disease after the age of 6 months there are few, if any, neurologic changes beyond occasional mental retardation. The disease still advances more rapidly than in

adults and death may occur a few years after onset although most childhood cases survive into adult life

In the adult the majority of the slowly developing clinical manifestations are related solely to the effects of an increasing mass of Gaucher cells in the bone marrow spleen liver and lymph nodes and occasionally a few other tissues The typical case is often diagnosed when asymptomatic splenic enlargement is detected during a routine examination In time the continuing enlargement of the spleen leads to distressing pressure symptoms and liver enlargement and moderate lymphadenopathy will have been noted Concomitantly increasing pigmentation of the skin and frequently pigmented areas of scleral thickening (pingueculae) appear in the eyes Bone lesions may give rise to pathologic fractures and the additive effects of marrow and splenic involvement lead to significant pancytopenia necessitating splenectomy Eventually marrow replacement by Gaucher cells may preclude adequate blood cell formation and as liver and lymphatic involvement increase there is general deterioration in nutritional state and death results from intercurrent infection The natural history of Gaucher's disease will no doubt be altered by the increasing use of antibiotics and the survival of both children and adults improved

### THE GAUCHER CELL

The typical cell in Gaucher tissues is a unique pathologic entity its presence makes possible an unmistakable diagnosis The peculiar feature of these large (20 to 100 microns) cells is the reticular pattern in the cytoplasm resembling nothing so much as wrinkled tissue paper or crumpled silk (Fig 19 1) This irregular streaked appearance is most obvious in fixed preparations stained with Mallory's trichrome connective tissue stain and is especially well demonstrated by phase microscopy [8] Strong acid phosphatase activity is demonstrable histochemically [23a] The many studies relative to the cytology and histogenesis of the Gaucher cell have been comprehensively reviewed by Pittaluga and Goyanes [27] and Block and Jacobson [28]

It is believed that the Gaucher cells arise from the reticulum cells [3 10 23-25] and possibly osteoblasts and fibroblast like spindle cells adjacent to bone spicules in the marrow [28] The absence of reactivity with the usual fat stains and the strong staining by the periodic acid-Schiff reaction [34-37] lend support to the generally accepted assumption that the cells are filled with cerebroside The PAS positive material is difficult to extract with lipid solvents [34-37] a phenomenon which has been interpreted as strong (protein) binding of the lipid within the cells [37] Using supravital techniques Erf studied Gaucher cells in various stages of development and reported the gradual transformation of the cytoplasm of the cells from a liquid to a semisolid state as presumably

cerebrosides accumulated [33]. As the cells matured the mitochondria disappeared. The cells are seldom seen in mitosis and appear to divide mainly by nuclear cleavage [27-39]. Attempts to produce Gaucher cells by injection of cerebroside suspensions have been made [34, 40-44] and transient changes in reticulum cells having some of the typical features have been observed. None of the findings necessarily has direct bearing on the etiology of Gaucher's disease. Granulomatous changes do not

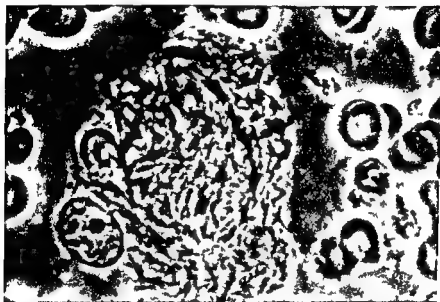


Fig 19-1 The Gaucher cell shown in supravital preparation of bone marrow biopsy. The size of the cell may be compared with that of the adjacent erythrocytes. (Photomicrograph courtesy of Dr. George Brecher, National Institutes of Health.)

accompany Gaucher cell formation, and histologic evidence of another primary process is absent.

The transformation of reticulum cells to Gaucher cells produces striking changes in several organs, and these, with their attendant functional disturbances, are worthy of comment.

#### SPLLEN

Widespread replacement of the normal architecture of the spleen by Gaucher cells is characteristic, and an enlarged, firm, nontender organ is palpable in nearly every case, with a few notable exceptions [26, 28, 38, 44]. Although gigantic splenomegaly in the later stages may produce considerable mechanical distress, perisplenitis and rupture are uncommon. The most important consequences are thrombocytopenia, leukopenia, and anemia related to hypersplenism.

The anemia is slight to moderate and usually increases with the duration of the disease. Few erythrokinetic studies have been made although a shortened erythrocyte life span has been suggested [45]. A moderate reticulocytosis is common [46] but presumably decreased erythropoiesis in the involved marrow as well as increased erythrocyte removal by the spleen both contribute to the anemia.

A hemorrhagic tendency is a frequent complication in the adult case and may be the presenting sign. That this is due to the frequent thrombocytopenia is indicated by the clotting studies which have been made. The morphology of the megakaryocytes and platelets is normal and splenectomy promptly causes the platelet count to rise. A rare case has been reported in which prothrombin time was increased associated with hypofibrinogenemia and deficits in convertin and accelerin [47]. The tendency to spontaneous bleeding is expressed in numerous hemorrhagic infarcts and may account for some of the marked hemosiderosis in the tissues of the adult cases.

#### LIVER

The liver is consistently and diffusely involved, the masses of Gaucher cells appearing grossly as white translucent spots. The pressure of these expanding masses destroys parenchymal cells and the lobular architecture is distorted with an accompanying increase in connective tissue. Groups of cells may surround the central and hepatic veins and obstruct the liver capillaries [48-50]. Moderate hepatomegaly is present in the majority of patients [46] and ascites may occasionally be present [49]. There are no characteristic changes in liver function test results [46, 50, 51] or in serum protein concentrations [52]. In a rare case the serum alkaline phosphatase level may be elevated in association with severe osseous involvement [53].

#### BONE

The development of abundant Gaucher cells—often peculiarly fascicular or spindle shaped—throughout the marrow and cancellous bone is common and may lead to spectacular nearly complete replacement of marrow accompanied by pain, pathologic fractures and vertebral collapse [5]. The destruction of bone and joint cartilage is accomplished through pressure or ischemic necrosis; true invasion of cortex, periosteum or cartilage has not been observed [5]. The phalanges, long bones, vertebrae, ribs or pelvis are involved much more commonly than the skull although mandibular lesions have been noted. Expansion of the cortex at the lower end of the femur producing a radiolucent area with the contour of an Erlenmeyer flask is responsible for a characteristic deformity. Destruction of the vertebral bodies produces a thin, shortened cylinder often ending with complete spinal fusion and gibbus forma-

tion Bone pain may be accompanied by erythema and swelling and sinuses with Gaucher cells in the drainage [53], may develop simulating osteomyelitis

Junghagen's thorough study published in 1926 contains one of the most extensive descriptions of the radiologic changes in the bones in Gaucher's disease [54] Relief of acute bone pain has been reported after x ray therapy, as has radiologic improvement in a large cystic lesion [55] Radiation has also failed to influence the development of a typical lesion in bone [8]

#### LYMPHOID TISSUE

Gaucher cells commonly occupy the area of the general reticulum outside the lymph sinuses [30] in the deep abdominal and thoracic lymph nodes, they frequently cause enlargement of peripheral nodes as well Peripheral lymph gland enlargement is much more common in infantile than in adult cases [6-9] There is also present an increased accumulation of iron containing pigment in the nodes most being present in reticular macrophages but some unequivocally present in the Gaucher cells [27-30] Although the degree of lymph node involvement is quite variable biopsy of this tissue is useful for diagnosis

#### EYES

Brill [56] in his early description of two involved siblings first called attention to the pingueculae in Gaucher's disease In about 25 per cent of patients there are present bilaterally on the sclera striking yellow brown wedge shaped patches whose bases abut the cornea with the apices extending to the canthi [46] They are asymptomatic and require no treatment

The pingueculae in Gaucher's disease may occur at any age beyond childhood and are larger and darker than those commonly seen in other wise normal individuals after middle age In one instance [57] small collections of large epithelial cells with foamy cytoplasm which did not take conventional lipid stains have been described in a Gaucher pinguecula Typical Gaucher cells have not been described in these lesions

#### SKIN

Characteristic diffuse yellow brown pigmentation develops on the exposed surfaces in many adult patients It is sometimes localized or unilateral [9] or it may be symmetrically distributed on the lower legs [58] or over the face associated with a characteristic malar flush Diminution of pigmentation following splenectomy has occasionally been reported [59] Definitive studies on the pathogenesis of the pigmentation are

needed Gaucher cells have not been found in the skin although increased iron containing pigment [58] and melanin [60] have been reported

### OTHER ORGANS

As previously mentioned infantile patients may show heavy Gaucher cell infiltration in tissues other than spleen liver lymph nodes and bone marrow Heavy involvement of the lung may be present producing miliary opacities in the pulmonary x ray [7] Changes in the lung parenchyma with Gaucher cells in the sputum in one case have been reported in two adults [61] Rarely in adults the adrenal gland [62] thyroid [59] and kidney [63] may also show histologic lesions without evidence of functional impairment

### NEUROLOGIC LESIONS

Present knowledge of the nature of the process occurring in the brain of infantile cases and its relationship to altered neurologic function is highly inadequate The histologic changes reviewed by several authors [2 37 64-68] consist of (1) nonspecific alteration in the cytoplasm of the ganglion cells with loss of Nissl substance and sometimes [2 68] vacuolization and ballooning of the cell body and (2) glial proliferation Some evidence of demyelination has been reported [69] Only on rare occasions [69 70] have Gaucher cells been seen in the brain and the e were localized to the hypophysis and hypothalamus or to the perivascular areas There are two reported cases in children in which neurologic function was abnormal but no histologic changes could be found in the brain after careful search [2 71] Furthermore as discussed in more detail later the cerebroside content of the brain has never been demonstrated to be elevated Also the cerebroside present is of the normal type as opposed to the altered cerebroside in the viscera It should be noted that while lesions in the brain are classically considered to be restricted to infants altered ganglion cells may be seen in an occasional adult patient [70] Exhaustive microchemical dissection of the Gaucher brain is sorely needed

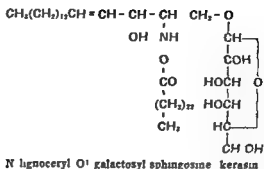
Before discussion of the chemical changes in Gaucher's disease a review of what is known about cerebroside and their metabolism may be helpful

### CEREBROSIDES

#### STRUCTURE AND OCCURRENCE

Cerebrosides consist of ceramide (N-acyl phingone) and hexose The molar ratios of phingone base fatty acid and hexose are 1:1:1 as indicated by the accompanying structural formula





These compounds were first isolated from brain and given the name cerebrosides by their discoverer, Thudichum [20]. Although Thudichum described their general structure correctly, it is through the subsequent work of a number of other workers including notably Thierfelder, Levene, Rosenheim and Klenk that the nature of the fatty acid and hexose moieties has been gradually understood.

A number of other generic names for these compounds has been used such as galactolipids, galactosides, glycolipids (for glucocerebrosides), sphingoglycosides and glycosphingosides. None of these is more useful in distinguishing the compounds having phingosine fatty acid and hexose in equimolar amounts from other sugar-containing sphingolipids found in tissues (see below). The use of trivial names for various cerebrosides based on the nature of the fatty acid component, e.g., cerebrin for the compound containing cerebronic acid, has long been a common practice. It is becoming less frequent largely because of increasing awareness of the complexity of the fatty acids present in tissue cerebrosides and supposedly pure preparations of a given compound.

### *Sphingosine Moiety*

The chemistry and biochemistry of sphingosine base have been discussed previously (Chap. 17). Many of the studies of sphingosine structure have utilized base isolated from brain and spinal cord cerebrosides. It is established with near certainty that in naturally occurring cerebrosides the C<sub>4</sub>-C<sub>5</sub> double bond of sphingosine has the *trans* configuration [72-73] and the substitutions at C<sub>2</sub> and C<sub>3</sub> the *erythro* configuration [74-75]. A large fraction of the base in naturally occurring cerebrosides is the saturated compound dihydro sphingosine [76].

### *Fatty Acid*

The fatty acid joined to sphingosine in acid amide linkage in cerebrosides is of variable composition and has been the subject of intensive research and lively debate since the discovery of these compounds. Fortunately a mist of confusing terminology generated by these activities

has largely disappeared<sup>1</sup> and the recent application of gas chromatography and improved techniques for separating cerebrosides has greatly increased available information in this area.

By 1930 it was well established that the major acids in brain cerebrosides contained 24 carbon chains. The first two cerebrosides isolated by Thudichum, kerasin and phrenosin (cerebron [80]) were found to contain primarily lignoceric acid  $[\text{CH}_2(\text{CH}_2)_{22}\text{COOH}]$  [81-83] and phrenosinic (cerebronic) acid  $[\text{CH}_2(\text{CH}_2)_{21}\text{CHOHCOOH}]$  [84-86] respectively. To these were subsequently added nervon containing nervonic acid  $[\text{CH}_2(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_{13}\text{COOH}]$  [86] and oxynervon containing oxynervonic acid  $[\text{CH}_2(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_{13}\text{CHOHCOOH}]$  [85]. It was soon established that the most important acid was probably cerebronic [84].

It subsequently became clear, however, that the quantitative estimates of the content of particular cerebrosides might be in error because of preferential solubilities introduced by the techniques of isolation and that many homologues of the  $\text{C}_{24}$  acids are present varying in chain length from at least  $\text{C}_{18}$  to  $\text{C}_{28}$  [78-87-93].

The studies of Hashimoto and Radin [93-94] are the most complete of the available analyses of the fatty acid components. They have demonstrated that rat brain cerebrosides contain a broad spectrum of acids. The saturated  $\alpha$  hydroxy acids constitute the major component. In this group the  $\text{C}_{24}$  acids predominate but  $\text{C}_{22}$  and  $\text{C}_{26}$  acids are also present in appreciable amounts. The saturated normal acids (mainly  $\text{C}_{24}$  then  $\text{C}_{22}$ ) are next in order of concentration. The unsaturated acids, both normal and hydroxylated and again mainly  $\text{C}_{24}$  in length are minor components.

The relative concentrations of all these classes of cerebroside fatty acids vary with maturation of the brain. The major change in postnatal development of the rat brain is an increase in the saturated hydroxy acids associated with a fall in the unsaturated acids [94].

### Hexose

The hexose in brain cerebrosides, called cerebrose by Thudichum, was first identified as D galactose by Thierfelder [95] and Brown and Morris [96]. All subsequent analyses have indicated that practically all (>90 per cent) of the hexose in the cerebrosides contained in a variety of normal tissues from several species is galactose [92-97-100]. Small amounts of glucose have been reported in normal spleen and brain cerebrosides [9-98-101]. The hexose present in cerebroside isolated from the liver in Gaucher's disease is significantly different from that present in normal tissues (see below).

<sup>1</sup> For the reader interested in the historical development of cerebroside chemistry several other references are suggested [1-9].

The hexose is linked to sphingosine through the C 1 hydroxyl [107 103] Since cerebroside is not a reducing substance the linkage includes the aldehydic carbon of the sugar It has not yet been established whether the glycosidic linkage is through an  $\alpha$  or  $\beta$  anomeric bond Psychosine (galactosphingosine) prepared from normal brain glycolipid [104] and glucocerebroside from Gaucher spleen [22] have been hydrolyzed by  $\beta$  galactosidase (emulsin) Others have found enzymatic reactions favoring an  $\alpha$  linkage [105] Spectrophotometric evidence for the  $\beta$  linkage in Gaucher glucocerebroside has also been recently reported [106]

#### PROPERTIES AND ISOLATION

Cerebrosides are alcohol soluble acetone insoluble optically active compounds which can be crystallized in a relatively pure state Their determination requires lipid extraction with careful purification followed by hydrolysis and quantitative estimation of the sugar moiety Quantitative methods for cerebroside determination have been recently reviewed in detail by several authors [107-109] Chromatographic techniques for purification have also been developed [93, 107 109 110]

The major problem in analysis of cerebrosides is their isolation from other glycolipids These latter include not only gangliosides which are distinguished by their content of hexosamine and sialic acid (Chap 17) but other neutral glycolipids which contain more than one mole of hexose per molecule Klenk and coworkers [97 111] have isolated cerebroside-like molecules from both beef spleen and human erythrocytes which contain about 40 per cent hexose including both galactose and glucose Klenk [100] has noted that these appear to be transition compounds lying between gangliosides and cerebrosides and that they may signify an important pathway of interconversion between the several classes of glycolipids

Uzman and Rumley [112] have studied changes in the content of cerebrosides and water soluble glycolipids in the developing mouse brain The hexose and neuraminic acid contents of the latter compounds undergo changes during development at a time when cerebrosides are actively being laid down in the brain These changes are also interpreted by the authors as suggesting a precursor role for water soluble glycolipids in cerebroside formation In this regard further characterization of the ceramide portion of such lipids is needed Klenk and Rennkamp [97] found in bovine tissues that the fatty acids in gangliosides and cerebrosides were similar in the spleen but dissimilar in the brain

#### CEREBROSIDE CONTENT OF TISSUES

The difficulties in obtaining pure cerebrosides have led to conflicting and sometimes meaningless reports of 'cerebroside' content of various

tissues. The methodology must be carefully scrutinized in interpreting these data particularly those found in the older literature.

In the brain the problem of separate determination of cerebrosides is of special importance because of the high content of gangliosides and other water soluble glycolipids. The values obtained up to 1956 for cerebroside content of brain have been summarized by Edgar [108]. A content of about 5  $\mu$  gm per 100 gm dry weight has been reported for human gray matter [113-115] and adult whole brain [116] and three times this concentration in white matter [113-115].

These estimates of cortical cerebroside content are probably too high. More recent analyses indicate that cerebrosides are present in very low concentration in gray matter [117]. Cerebroside is one of the characteristic lipids of the myelin sheath; therefore not only is it present mainly in white matter but its content increases as myelination proceeds in maturation of the brain [94, 112, 115, 118-121]. In a careful study of the glycolipid content of mouse brain combined with histochemical estimate of myelin content, Uzman and Rumley [112] found neither myelin nor cerebroside present at birth. Cerebrosides became detectable at 10 days of age and then rose very sharply in concentration reaching a maximum in animals about 42 days old. Similar findings have also been reported for the rabbit brain [121]. Differences with age in the cerebroside content of brain are also indicated by studies in the rat by Kishimoto and Radin [94] who found the content to rise from 0.34 gm per 100 gm wet weight of tissue to 1.1 gm in animals approximately 23 and 418 days old respectively. Less precise figures for changes with maturation are available for the human brain. The content of cerebrosides has been reported to increase until about age 16 after which it declines slightly [120].

In non nervous tissue the concentration of cerebrosides is much lower. The highest concentrations in viscera (on the order of 0.5 gm per 100 gm dry weight) are found in lung and spleen [116]. Many values for the concentration of cerebrosides in plasma have been reported. In recent careful analyses, Svennerholm and Svennerholm [122] found normal values to range from 3.0 to 5.7 mg per 100 ml. The content of gangliosides was slightly lower. According to Burt and Royster [123] the cerebroside content of leukocytes (rabbit) is considerably higher than that of erythrocytes. The latter contain about 30 mg cerebroside (neutral glycolipid) per 100 gm fresh tissue.

#### BIOSYNTHESIS OF CEREBROSIDES

Relatively few studies of cerebroside biosynthesis have been made and these have been mainly confined to brain. In this tissue following the intraperitoneal injection of the substrate the radioactive carbon atoms from acetate  $1\text{-C}^{14}$  and octanoate  $1\text{-C}^{14}$  [110, 124], galactose  $1\text{-C}^{14}$  [125-126], glucose  $1\text{-C}^{14}$  [125, 127-129] and glucose  $6\text{-C}^{14}$  [128, 129] have been

found in neutral glycolipids (in most cases demonstrated beyond reasonable doubt to be cerebroside) Such incorporation *in vivo* has been demonstrated in several species, including the monkey rat and mouse

It is also established that either glucose or galactose is mainly incorporated into the cerebroside as a unit with most of the labeled glucose appearing as galactose in the cerebroside [127-129]

The rate of incorporation of either galactose or glucose into brain cerebroside depends on age and apparently is directly related to the rate of myelin formation In mice some synthesis is demonstrable at birth the maximum occurring at about 16 to 22 days [128-129] or very close to the time of maximum myelin formation suggested by chemical analyses during maturation in this species [112-119] In rats there is practically no synthesis up to 7 days The maximum rate of incorporation of hexose is reached at 14 to 16 days and is reduced shortly thereafter [127]

When labeled hexose is injected intraperitoneally into young rats there follows a rapid rise in the activity in brain cerebroside the maximum being reached in about 6 hr [127] or slightly later [126] The radioactivity then declines indicating active turnover of cerebroside In the 15 day-old rats Burton et al [127] noted that the cerebroside radioactivity fell to a third of the maximum value within 24 hr Radin et al [126] found that the rate of incorporation of labeled galactose into brain gangliosides was about the same as that for cerebroside The cerebroside sulfatides were labeled more slowly

It has been recently shown by similar techniques that the maximum rate of synthesis of brain gangliosides is achieved at an earlier age than that for cerebroside in rats [130] This is consistent with the earlier appearance of ganglioside in the brain and possibly with a precursor role of these compounds in cerebroside formation Moser and Karnovsky [128] have followed the incorporation of labeled glucose and galactose into both cerebroside and gangliosides in mice at different ages Cerebroside 'synthesis' appears to slack off with increasing age more pronouncedly than 'synthesis of gangliosides The specific activity of galactose in cerebroside moreover was greater than that in gangliosides As they point out it is extremely difficult to interpret such findings in terms of possible precursor-product relationships They do not provide support however for the conclusion that the ganglioside fraction as a whole forms a precursor pool for the formation of cerebroside

It is interesting that the incorporation of labeled hexose into brain cerebroside sulfatides is not associated with any appreciable rate of decay These substances increase during much of the life span [118] and may have important implications in the processes of aging in the central nervous system [126]

## STUDIES IN VITRO

Definitive studies of the synthesis of cerebrosides in cell free brain tissue have been made by Burton Sodd and Brady [127] All subcellular brain fractions are capable of incorporating galactose  $C^{14}$  into cerebrosides the greatest activity being associated with the microsomal fractions It is proposed that the pathways for both glucose and galactose incorporation into brain glycolipids involve the uridine nucleotides [127 131] with prior conversion of glucose to galactose [127]

The glycosyl transfer reactions have been described in detail in Chap 7 All the necessary enzymes have been demonstrated in brain [132-136]

For incorporation of galactose and glucose into cerebrosides the reactions as summarized by Burton et al [127] include

## Glucose Incorporation



## Galactose Incorporation



The position of UDP galactose as immediate donor (Reaction 5) is demonstrated by the failure of either galactose 1 P or galactose + ATP to be utilized unless UDP glucose is also present The conversion of glucose to galactose before incorporation is strongly suggested by the inhibition of utilization of glucose but not of galactose when the cell free particles are preheated UDP galactose 4-epimerase catalyzing Reaction 4 is heat sensitive Chromatographic evidence was obtained in these studies that the cerebrosides synthesized included N lignoceryl  $O^1$  and N-cerebronyl  $O^1$  galactosyl sphingosine [127]

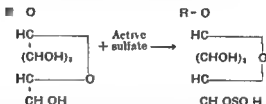
In Reaction 5 the acceptor for the hexose is indicated to be ceramide but this has not been proved Other evidence has been presented that the acceptor may be free sphingosine [128 131a] It should not be concluded that conversion of ceramide or sphingosine to cerebrosides through the uridine nucleotide pathway is necessarily the only or even the major route of cerebroside synthesis in all organs It should also be noted that some glucose can apparently be incorporated directly into cerebrosides [128 129]

Trams and Brady have demonstrated incorporation of both labeled galactose and glucose into cerebrosides by human spleen tissue slices obtained from a patient with Crücher disease and another with Nie

mann Pick disease [137] Rat liver apparently also may incorporate a very small amount of hexose into cerebroside [127]

### DEGRADATION AND UTILIZATION

Several theoretic pathways exist for cerebroside degradation or further utilization. One is conversion to cerebroside sulfatides.

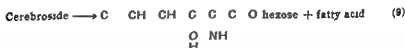


The lack of evidence for appreciable turnover of the sulphatides suggests that this would be mainly a unidirectional reaction [126]. In this regard it may be significant that the ratio of cerebroside sulfate to cerebroside increases with age in the brain [94]. Other possible reactions are conversion to polycerebroside (see below) or other water soluble glycolipids through further addition of hexose, hexosamine and sialic acid units. None of these reactions has been demonstrated and speculations that reactions in the opposite direction might be more important [100-112] have been previously mentioned.

Cerebroside may be degraded to (1) ceramide and hexose through hydrolysis of the glycoside bond (Reaction 8) and in theory to (2) psy-



choline and fatty acid through hydrolysis of the acid amide bond (Reaction 9)



The first reaction has been demonstrated in tissues; the second has not. It has been reported that small amounts of ceramide may be present in liver and spleen [138-139] but neither free ceramide nor psychosine has been reported in brain.

The production of ceramide (Reaction 8) is catalyzed by several types of hexoside hydrolases. Galactosidases such as are found in sweet almond emulsin [22] and extracts of *Escherichia coli* [105] split cerebroside. A "cerebrosidease" was first discovered in extracts of human spleen/brain.

and pancreas by Thannhauser and Reichel [140] They found that hydrolytic activity was greatly enhanced by activation of the extract with reducing substances such as hydrogen sulfide cysteine or reduced glutathione Their work has been confirmed by Iujino [141] who determined that the products of the reaction were ceramide and hexose Under the conditions employed the splenic extracts did not attack the N acyl linkage of ceramide No enzymatic hydrolysis of this bond has been studied Other evidence suggestive of cerebroside-splitting activity in nervous tissue is the disappearance of glycolipid which occurs during Wallerian degeneration [142] It has been observed that this process is accompanied by an increase in macrophages or Schwann cells about the affected peripheral nerve suggesting that these cells may contain the degradative activity [143]

Thus far too little is known of the manner in which cerebroside are broken down or converted to other products Although ceramide has been repeatedly discussed as a pivotal intermediate in sphingolipid synthesis [140] there have been no studies on relatively pure enzymes which reveal the equilibria and other conditions by which ceramide might be preferentially used for one or another sphingolipid

## CHEMICAL ABNORMALITIES IN GAUCHER'S DISEASE

### TISSUE CEREBROSIDES

The characteristic chemical alterations in Gaucher tissues involve the cerebroside content The alterations have two possibly three important features These are (1) an increase in total cerebroside concentration (2) accumulation of cerebroside containing abnormal sugar components and (3) possible alterations in the physical state and molecular form of the cerebroside Significant alteration in the content of other tissue lipids has not been demonstrated

#### *Cerebroside Content of Tissues*

In involved viscera the cerebroside concentration may be increased by tenfold or more [9 60 87 116] Extensive analyses of a case published by Ottenstein Schmidt and Thannhauser [116] and by Thannhauser [9] are shown in Table 19.1 The few quantitative analyses of brain tissue in which there were anatomic abnormalities [87 116] do not demonstrate any increase in cerebroside content although an increased content of polycerebroside in a Gaucher brain has been reported by Uzman (see below)

#### *Carbohydrate Content of Cerebroside*

The cerebroside in the viscera in Gaucher's disease contain mainly glucose rather than the galactose found in normal cerebroside



Whether an increase in galactocerebroside also occurs in this disease has been seriously questioned [98] but it should probably be accepted that it does. In Table 19.2 are summarized the reports in which some attempt was made to identify the cerebroside carbohydrate. The earlier studies relied mainly on the melting point of the osazone and fermentation studies. Differential color reactions have been frequently used and more recently paper chromatography and specific enzymatic reactions such as glucose oxidase [106]

TABLE 19.1 CEREBROSIDE CONTENT IN ORGANS FROM A  $1\frac{1}{2}$  MONTH OLD PATIENT WITH CAUCHER'S DISEASE

| Organ         | Total cerebroside content<br>mg per 100 gm dry weight |           |
|---------------|---|-----------|
|               | Caucher's   | Normal    |
| Spleen        | 1.44  | 0.1-0.5   |
| Liver         | 0.33  | 0.05-0.15 |
| Lung          | 1.01  | 0.1-0.6   |
| Kidney        | 0.54  | 0.1-0.7   |
| Pancreas      | 0.14  | 0.1-0.3   |
| Thymus        | 0.60  |           |
| Adrenal gland | 0.71  |           |
| Brain         | 3.21  | 4.0-6.0   |

Source: Abstracted from the data of H. Ottenstein et al. [116] also published by J. Thannhauser [9]. The normal values for brain were those obtained in a 1-day-old child and are undoubtedly lower than normal for the age of the patient with Caucher's disease.

The analyses reported up to 1950 were compiled and critically reviewed by Brante [98]. He also reported 4 cases in which glucose represented over 90 per cent of the sugar in the splenic cerebroside. Brante further concluded that in the instances where galactose was reported as the major sugar [16, 116, 144, 145] the methods may have been subject to error and that any increase in galactocerebroside was unproved. At least 12 more case studies have been subsequently reported. In 8 of these the spleen cerebroside contained practically only glucose. There are several important exceptions, however. Montreuil et al. [101] using paper chromatographic methods capable of separating glucose and galactose mixtures found 45 per cent galactose in the cerebroside. Farke [155] found equimolar concentrations of glucose and galactose after complete hydrolysis and lactose after partial hydrolysis. Woolf [156] who found mainly glucose after complete hydrolysis also reported finding paper chromatographic evidence of fructose, lactose and sucrose after only partial hydrolysis. In the cerebroside isolated in polymolecular form discussed below, Uzman found a preponderance of galactose. The

TABLE 19-2 THE NATURE OF CARBOHYDRATE IN CEREBROSIDES FROM GAUCHER TISSUES

| Case reference | Year | Tissue source                    | Cerebroside sugar |           |       |
|----------------|------|----------------------------------|-------------------|-----------|-------|
|                |      |                                  | Glucose           | Galactose | Other |
| [16]           | 1934 | Spleen                           |                   | +         |       |
| [144]          | 1933 | Spleen                           |                   | +         |       |
| [1]            | 1934 |                                  | +                 |           |       |
|                | 1940 | Spleen                           | +                 |           |       |
| [87]           | 1940 | Spleen                           | +                 |           |       |
| [8]            | 1940 | Brain                            |                   | +         |       |
| [8]            | 1940 | Liver lung spleen                | +                 |           |       |
| [1'5]          | 1941 | Spleen                           |                   | +         |       |
| [1'6]          | 1942 | Spleen                           | +                 |           |       |
|                |      | Liver                            | +                 |           |       |
| [1'7]          | 1942 | Spleen                           | +                 |           |       |
| [1'8]          | 1942 | Spleen                           | +                 |           |       |
| [149]          | 1946 | Spleen                           | +                 |           |       |
| [116]          | 1948 | Spleen liver other viscera       | +                 | +         |       |
|                |      | Brain                            |                   | +         |       |
| [118]          | 1948 | Liver spleen brain other viscera |                   | +         |       |
| [116]          | 1948 | Spleen                           | +                 |           |       |
| [116]          | 1948 | Spleen                           | +                 |           |       |
| [116]          | 1948 | Spleen                           | +                 | +         |       |
| [116]          | 1948 | Spleen                           | +                 | +         |       |
| [116]          | 1948 | Erythrocytes                     |                   | +         |       |
| [116]          | 1948 | Erythrocytes                     |                   | +         |       |
| [150]          | 1948 | Spleen                           | +                 |           |       |
| [1'1]          | 1950 | Spleen                           | +                 |           |       |
|                |      | Brain                            |                   | +         |       |
| [98]           | 1951 | Spleen                           | +                 |           |       |
|                |      | Liver                            | +                 |           |       |
|                |      | Erythrocytes                     |                   | +         |       |
| [98]           | 1951 | Spleen                           | +                 |           |       |
| [98]           | 1951 | Spleen                           | +                 |           |       |
| [98]           | 1951 | Spleen                           | +                 |           |       |
| [15]           | 1951 | Spleen                           |                   | +         |       |
| [1'9]          | 1953 | Brain                            |                   | +         |       |
|                |      | Spleen                           | +                 | +         |       |
| [101]          | 1953 | Spleen                           | +                 | +         |       |
| [15]           | 1954 | Spleen                           | +                 | +         |       |
| [15]           | 1954 | Spleen                           | +                 |           |       |
| [155]          | 1954 | Spleen                           | +                 | +         |       |
| [156]          | 1954 | Spleen                           | +                 | +         |       |
| [156]          | 1954 | Spleen                           | +                 | +         |       |
| [15]           | 1954 | Spleen                           | +                 |           |       |
| [156]          | 1954 | Spleen                           | +                 |           |       |
| [15]           | 1958 | Spleen                           | +                 |           |       |
| [156]          | 1958 | Spleen                           | +                 |           |       |

Sugar identified after methods as Gaucher lipoprotein [15] or polycerebroside [153]

reported differences in carbohydrate moiety have no correlation with the age of the patients

In the case reports of Ottenstein et al [116] the question of galactocerebroside identification is especially important. These authors who used fermentation in addition to the *o* zone for identification found increased glucocerebrosides in one case and increased galactocerebrosides in an involved sibling. If they are not due to subtle errors in experimental technique these findings and those indicating polysaccharides in the Gaucher cerebroside tend to diminish the implications of a specifically abnormal compound in the involved tissues.

In viscera in Gaucher's disease nevertheless the usual substitution of glucose for galactose in the excessive cerebroside may constitute a key biochemical abnormality. The interesting findings of disaccharide need further evaluation. It is important in this regard that most of the cerebroside carefully isolated from Gaucher tissues have contained enough reducing substance (after complete hydrolysis) to account for only a single hexose unit in the molecule.

The brain cerebroside examined so far have contained galactose only.

### *Polymolecular Substances*

Uzman has reported several interesting studies of the form in which cerebroside may be present in the Gaucher spleen and brain [152-153]. These include the isolation of the cerebroside in a "Gaucher lipoprotein" and by another approach as "polycerebroside".

In the first instance spleen was extracted in aqueous media and after repeated precipitation and resolution 70 per cent of the total cerebroside was retained in solution in saline. This water soluble material contained about 40 per cent protein and 60 per cent lipid, almost all of which was cerebroside. No lipid phosphorus or free amino nitrogen was present. A molecular weight for this lipoprotein obtained by osmometry was about 320 000. As estimated from the difficulty with which the protein was freed of lipid by solvent extraction the lipid-protein binding was quite strong. No comment was made about the possible presence of the lipoprotein in normal tissues.

In the second study [153] water soluble glycolipid was isolated from Gaucher spleen and brain. This material was more soluble in acidified aqueous media than ordinary cerebroside but it did not contain the hexosamine or sialic acid characteristic of ganglioside or 'mucolipid' (Chap. 17). Cerebroside was liberated by hydrolysis and these contained a galactose to glucose ratio of about 4:1. This 'polycerebroside' migrated on electrophoresis as a single boundary with a negative charge. The absence of phosphorus or sulfate suggested this charge may have been due to free carboxyl groups. The molecular weight was estimated by diffusion and osmometry to be about 30 000.

Uzman reported finding polycerebroside in normal brain in small quantities but not in the spleen in the absence of Gaucher's disease [153]. He suggested that polycerebroside was not necessarily a polymer of repeating cerebroside units and that it could be a precursor for the Gaucher lipoprotein which he had earlier described.

Confirmation and extension of these interesting findings are awaited. Klenk, in reviewing his studies of the Gaucher spleen [100], states that the water soluble polycerebrosides described by Uzman could be isolated in only small amounts (46-176 mg per 100 gm of wet tissue). He makes the further observation that the polycerebrosides resemble the glycolipids in bovine spleen [97] and suggests that the Gaucher cerebroside might be liberated enzymatically from water soluble glycolipids like polycerebrosides and gangliosides.

Other possible abnormalities in cerebroside structure such as in the sphingosine or fatty acid portions of the molecule have not been established in Gaucher's disease. Behenic acid (saturated  $C_{22}$ ) has been twice reported as a major component of the excessive cerebroside in the spleen, liver and lung [87, 106]. In the brain of one of these cases an infant with ganglion and glial cell alterations the major fatty acid in the brain cerebroside was cerebronic. This was interpreted by Klenk [87] as suggesting that in Gaucher's disease the involved tissues contained cerebroside with the fatty acid moiety normally characteristic for that tissue.

#### *Acid Phosphatase*

Striking increase in acid phosphatase activity in plasma and tissues is now a well established phenomenon in Gaucher's disease. Tuchman et al [23] were the first to report increased activity in plasma, noted as a chance observation. They subsequently investigated 17 more cases and found in every one increased activity against phenylphosphate in acid medium (7 to 14 Gutman units as compared with the normal range of 4 to 5 units) [158]. When  $\beta$  glycerophosphate was employed as substrate (Bodansky method) activities were lower and many were within normal limits. In contrast to the prostatic enzyme [159] the plasma acid phosphatase in Gaucher's disease is not inhibited by L-tartrate [158]. Neither is it inhibited significantly by copper ions or by formaldehyde as is the normal erythrocyte phosphatase [159].

Crocker and Landing [23a] have recently reported extensive studies

Marinetti et al [14] have recently published detailed analyses of cerebroside isolated from the spleen of a 50-year-old patient with Gaucher's disease. The cerebroside concentration was 0.89 gm per 100 gm of fresh tissue. Glucose appeared to be the only sugar present. The hexose was shown to be attached to the primary hydroxyl group of sphingosine. As determined by gas chromatography the major fatty acids were lignoceric, behenic and palmitic.

initiated years ago with the observation by Farber of marked acid phosphatase activity in the Gaucher cells themselves. All histiocytic cells have some weak acid phosphatase activity but the degree of hyperphosphatasia present in Gaucher tissues is not found in other conditions associated with enlarged macrophages such as Niemann Pick disease [23a]. With the possible exception of a single case [160] the plasma phosphatase is also normal in Niemann Pick disease (Chap. 18). It should be noted that one metabolic disease of bone—osteopetrosis—is also associated with acid hyperphosphatasia [161–23a].

Crocker and Landing [23a] are of the opinion that the increased enzyme activity in plasma has its origin in the Gaucher cells; it is not dependent upon the presence of the spleen. They also believe that the hyperphosphatasia may be an important clue to the etiology of the disease. Splenic acid phosphatase has been found active against a large variety of substrates with maximum activity against nucleoside triphosphates such as ATP [23a].

### *Blood Lipids*

There are no characteristic changes in the serum lipids in Gaucher's disease. Most importantly, no increase in cerebroside has been found. Thannhauser, using the methods developed by Svennerholm and Svennerholm [122], found 1.83 mg cerebroside per 100 ml plasma in a normal male and 1.23 and 1.83 mg per 100 ml in two adults with Gaucher's disease [9]. An increase in lipid nitrogen was reported in several cases by Bloem et al. [58] but this material was not further characterized. The nature of the hexose in the circulating cerebroside in Gaucher's disease has not been investigated.

## ETIOLOGIC POSSIBILITIES

It is all too apparent that present knowledge about normal glycolipid metabolism is inadequate to bear the burden of excessive speculation about the basic abnormality in Gaucher's disease. There may even be more than one biochemical lesion of importance. Several aspects of the disease deserve further comment with respect to etiology.

### TRANSPORT OF CEREBROSIDES

It is most probable, as Thannhauser was among the first to emphasize [9], that the excessive cerebroside is made by the Gaucher cells or their normal appearing neighboring reticulum cells. The normal concentration of cerebroside in extracellular fluid offers no evidence of increased transport from a remote site in this manner. It cannot be denied, however, that increased erythrocyte breakdown might account for considerable amounts of the cerebroside collecting in reticuloendothelial tissues. This was early

suggested by Marchand [12] and is supported by the accumulation of hemosiderin adjacent to and in the Gaucher cells and by the clinical impression that splenectomy results in improvement in some parameters other than hematologic ones. To be sure the absence of Gaucher cells in other types of hemolytic anemias, the paucity of hemosiderin in the tissues in infantile cases and above all the presence of abnormal cerebroides in the viscera mitigate against the primary importance of this mechanism. The latter objection may not prove to be valid however when more information about the glycolipid content of normal and Gaucher erythrocytes is obtained.

#### DEFECTIVE CEREBROSIDE METABOLISM

The appearance of glucose in the Gaucher cerebrosides found in the viscera is undoubtedly a most important clue. The finding of Trams and Brady [137] that a Gaucher spleen was capable of incorporating either galactose or glucose into cerebrosides *in vitro* is significant if not too surprising. There is no clinical evidence of a general defect in either galactose or glucose metabolism such as that in galactosemia although the individual glycosyl transfer reactions might well be studied in Gaucher tissues. More data will need to be compiled concerning the relative rates of hexose utilization in normal and involved tissues [137].

Whatever the status of glycolipid synthesis in Gaucher's disease it is evident that there is some step in the degradation or elimination of cerebroides which is disastrously rate-limiting. Beyond the previously mentioned studies with crude preparations of cerebrosidase the pathways for utilizing cerebrosides are unknown. It would be interesting also to know the ability of the normal complement of dissimulative enzymes to utilize glucocerebrosides as compared with galactocerebrosides.

The suggestion of abnormal polycerebrosides in Gaucher tissues and the possible interrelationships between the several classes of glycolipids which have been suggested [100, 112, 153] also raise an intriguing possibility demanding much further work. This is the accumulation of glucocerebrosides as end products of excessive synthesis and degradation of gangliosides or the more complex neutral glycolipids by the following type of reaction:



The relationship of such a mechanism to the possible presence of protein having an abnormally high affinity for cerebroides [142] also requires clarification.

The possibility that the hyperphosphatasia characteristic of Gaucher tissues might lead to degradation of phospholipid with diversion of substrate to cerebroides has been considered but left unsupported by the

finding of normal amounts of phospholipid in the  $\pi$  tissues [23a] Future work may supply a key to etiology by disclosing a more subtle effect of the phosphatase on cerebroside metabolism

Finally no aspect of Gaucher's disease is less well understood than the changes in the nervous system The dichotomy between the types of cerebroside found in the brain and viscera the lack of good evidence for any remarkable degree of abnormal myelination although a general defect in cerebroside metabolism is supposedly present and the reason for the changes in the ganglion cells will all no doubt become clear when more of the normal metabolic pathways are unraveled

## DIAGNOSIS

The diagnosis of Gaucher's disease can be made for practical purposes by the finding of the typical cells in spleen liver bone marrow lymph node or other tissues The relative merits of various tissues as a biopsy site have been discussed at length by Block and Jacobson [28] As discussed below there may be a "carrier" state of the disease in which the finding of immature appearing cells may be the only abnormality

Although the finding of Gaucher cells leaves little doubt concerning the diagnosis in every case attempt should be made to ascertain an elevation of cerebroside in the involved tissues and to determine chemically the nature of the cerebroside present A serum acid phosphatase determination should also be made using L-tartrate inhibition or similar means to differentiate the enzyme activity from the prostatic enzyme

The characteristic nature of the skin pigmentation pinguiculae and x ray changes in bones combined with other clinical features may help form a presumptive diagnosis but it should always be supported by histologic and chemical studies

## TREATMENT

There is no specific treatment Splenectomy must often be performed because of mechanical discomfort or the hematologic effects of hyper splenism It is followed by a prompt rise in platelets and leukocytes which may persist for years The increase in red cell mass which also occurs is often transient [9] Occasional improvement in pigmentation and decrease in pinguiculae have been reported after splenectomy [59] Cortisone may reduce capillary fragility or induce a slight rise in platelet count preoperatively [163]

Irradiation of the spleen is of no value in massive splenomegaly [8] It is of great value in reducing bone pain [9] Its use must be carefully tempered by consideration of the effect it will also have on the surviving bone marrow Bone pain may also respond well to steroid therapy [9]

Gaucher's disease is not incompatible with successful pregnancy [163-164]. An increase in abortions and stillbirths has been considered typical of the disease [165].

## GENETICS

The occurrence of Gaucher's disease in siblings was observed in the second case report to appear in the literature [189, 194]. Since that time ample evidence has accumulated to indicate that the condition is familial sibships in which five of six [166] and four of five [6] members were involved have been reported.

There is an apparent preponderance of Jews among reported cases although many have been reported in which Jewish ancestry was not present involving Caucasians, Indians [167], Filipinos [168-169], Mexicans [170], Japanese [171] and Negroes [172]. The sex distribution is about equal [4, 165].

There is no present means of determining whether all reported cases of Gaucher's disease represent a single mutation causing an identical biochemical lesion. There is some reason for suspecting they do not considering a recent review of the genetic aspects of some 110 cases by Hsia et al. [173]. They concluded that clinically Gaucher's disease may fall into at least two groups with different modes of transmission: (1) autosomal recessive in the majority of cases and (2) autosomal dominant one of the few well detailed examples of which is presented by them. Hsia et al. also add a possible third type of mutation based on the observation that if neurologic abnormalities are present they appear in all siblings affected with Gaucher's disease.

Most familial cases which have been adequately described have shown a frequency of involvement, lack of sex discrimination or consanguineous ancestry consistent with an autosomal recessive transmission. The most elaborate analysis of a pedigree of this type is that made by Herndon and Bender [172] of 5 cases in a closely related Negro sibship in North Carolina. These 5 cases had 17 siblings, 11 definitely normal and 6 who died in infancy of unknown causes. The apparent ratio of involvement in surviving siblings was therefore very close to 25 per cent. The authors concluded that transmission as a dominant trait with reduced penetrance could be excluded and that there was no evidence for sex linkage.

A dominant mode of inheritance was suggested by Groen [165] although in his review and 10 case presentations there were no cases providing unequivocal support for this mode. In 23 of 31 families Groen found that only one generation was involved; he emphasized the relative rarity of vertical transmission. He considered this to be due not only to decreased survival of patients into the reproductive period but also to increased frequency of stillbirths and abortions in marriages where



one partner has Gaucher's disease Groen suggested the possibility of anticipation or earlier development of the disease in succeeding generations until it extinguishes itself in infantile death Hsia et al [173] find the outlook for reproduction in involved families less gloomy Their analysis supporting several modes of transmission reinforces the need for determining the specific biochemical trait or traits and the determinants of their expression before the genetics of Gaucher's disease is clarified

### DETECTION OF THE TRAIT

A significant observation made by Groen has since been repeated by Stransky et al [168 169] This is the finding of small or 'young' Gaucher cells in the marrow of a parent of an involved child Some of these parents have been noted to have had pingueculae or very slight splenomegaly but their general health has been good In each case a normal bone marrow biopsy was obtained in the opposite parent This possible means of detecting a carrier state in Gaucher's disease needs to be explored in families of all involved cases

Elevated acid phosphatase in plasma has not been found in relatives of patients with Gaucher's disease [23a 162] This test however should continue to be performed on relatives until its implications are established The possibility that erythrocyte cerebroside may be abnormal in carriers does not appear to have been explored The development of a better biochemical test for heterozygosity (assuming recessive inheritance) is urgently needed the ability to predict satisfactorily the hazard of transmission of this crippling disease would be of inestimable value

### SUMMARY

1 Gaucher's disease is a rare familial disorder characterized by accumulation of cerebroside compounds containing sphingosine fatty acid and hexose in equimolar amounts in reticuloendothelial cells The storage cells possess a bizarre and characteristic appearance Their increasing mass is responsible for most of the clinical manifestations which include hepatosplenomegaly lymphadenopathy and bone lesions due to expansion of involved marrow

2 The disease may be detected at any age When it develops in infancy the course is malignant and further characterized by severe neurologic abnormalities The *et a* are associated with nonspecific changes in ganglion cells Cases developing in childhood may also lead to an early death or may progress slowly as in the adult form In the latter hematologic abnormalities associated with hypersplenism skin pigmentation and pingueculae usually develop

3 The involved tissues with exception of brain have a greatly in

creased content of cerebrosides. In most cases the cerebrosides are abnormal in that they contain glucose instead of the usual galactose. In several cases abnormal polycerebrosides or lipoprotein bound cerebrosides have been reported. The biochemical basis for the increase in cerebrosides is not known.

4. Elevated plasma acid phosphatase activity which is not inhibited by L-tartrate in contrast to the prostatic enzyme has been reported in all cases tested so far. The etiologic implications of this finding are unknown.

5. There is evidence that the disease may have several modes of transmission. The majority of cases appear to be inherited as an autosomal recessive trait. A few are consistent with autosomal dominant transmission. The biochemical homogeneity of the responsible trait remains to be established.

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Part Five

Disorders of Steroid  
Metabolism

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## Chapter 20

# The Adrenogenital Syndrome

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Gordon M. Tomlins and Joseph S. McGuire Jr

The adrenogenital syndrome is usually manifested by pseudohermaphroditism of females and premature virilization of males and may occur spontaneously or may result from various endocrine disorders such as tumors of the adrenal glands or gonads. The most frequent cause is congenital adrenal hyperplasia. Virilization of females and premature maturation of males are arresting clinical phenomena and individuals with the adrenogenital syndrome have attracted considerable medical interest.

Although many patients in earlier reports may have had the adrenogenital syndrome, the first convincing descriptions were by Otto in 1816, Cricchio in 1866, and Marchand in 1891 (cited by Bulloch [1]). In 1872 Ogston [2] described three female pseudohermaphrodites with bilateral adrenal hyperplasia, and in 1910 Aspert [3] published an important review in which the effects of adrenal hyperplasia were classified according to age of onset, a classification that is still of use. Callan can be credited with naming the syndrome *le syndrome génitosurrénal* in 1912 [4], and Callan in the same year [5] reported several patients with the adrenogenital syndrome, four of whom were boys.

### CLINICAL OBSERVATIONS

The syndrome of which there are several distinguishable forms is caused by heritable defects in the biosynthesis of steroid by the adrenal gland. The defects result directly and indirectly in the overproduction of certain steroids, some of which are androgenic. The cardinal sign of this syndrome is virilization, but the clinical manifestations depend upon the genetic cause, age of onset, and type and degree of the metabolic defect [6-12].

The patients can be conveniently divided into four groups according

to etiology and clinical expression (1) virilization (the major form) (2) virilization with adrenocortical insufficiency, (3) virilization with hypertension (4) virilization with adrenocortical insufficiency and hypertension

The age of onset of virilization is most commonly prenatal but is probably not before the fifth month of embryonic development [13]. Females exhibit a variety of malformations of the genitalia and sex determination is sometimes difficult because affected females resemble male pseudohermaphrodites. Chromosomal sex can be determined by skin biopsy [14] or by examination of oral mucosal smears [15-16]. The clitoris is enlarged and may enclose the urethra for a variable distance. Rarely the urethra may be completely penile [17] but more commonly there is a groove on the ventral surface of the clitoris which joins the urogenital sinus. The labia minora are small or absent. The labia majora are hypertrophied, rugose, pigmented and partially fused posteriorly (Fig. 20-1). Virilization likewise takes place in males but is often overlooked for variable periods because of its isosexual nature. Both sexes show accelerated somatic maturation. Bone age and growth are advanced. The epiphyses close prematurely and a growth deficit results. Pubic hair may appear in the second year, acne is common and there is pigmentation of the scrotum or labia and nipples. In the male the testes remain small and spermatogenesis does not occur [18-19]. Females usually have amenorrhea accompanying progressive virilization marked by male muscular development, male hair distribution, growth of beard and often temporal or frontal baldness. Breast development is slight and the voice is lowered in pitch.

When the adrenogenital syndrome is not present at birth but occurs in the prepubertal period, it is almost always due to adenoma or adenocarcinoma of the adrenal cortex. Progressive virilization takes place as in the congenital form and in the female is superimposed on normally developed genitalia.

When the syndrome occurs after puberty in females, menstruation becomes less regular and then ceases. Acne and hirsutism appear, the genitalia atrophy, the clitoris enlarges and the breasts regress. The voice deepens and the sex drive is often accentuated. The postpubertal onset of the syndrome has also been described in an adult male [20].

Less severe conditions which may be variants of the adrenogenital syndrome also occur in women. These individuals are hirsute and may have enlargement of the clitoris as well as menstrual and ovulatory disturbances [21-24].

#### ADRENOCORTICAL INSUFFICIENCY

In approximately half the patients with congenital adrenal hyperplasia there are in addition to virilism, adrenal insufficiency and associated

disturbances of salt and water metabolism as described first by Butler et al [25] and later by others [19 26-32]. The abnormalities characterized by urinary loss of sodium and chloride appear in the first week in half of those ultimately affected and by 7 weeks of age in almost all [33]. The infants are apathetic. Vomiting is the rule, diarrhea is common, and dehydration almost invariably occurs. The life-threatening complication is circulatory collapse comparable to that in Addisonian crisis. While the diagnosis suggests itself in females because of the associated genital malformations, it is often difficult in males. At birth most of the males have genitalia of normal size, but all show signs of virilization by 2 years of age. The fact that death occurred within the first 13 months in a large number of these children who did not receive specific treatment emphasizes the importance of prompt diagnosis in this group.

### HYPERTENSION

Another group of patients [34-35] with the adrenogenital syndrome has in addition to progressive virilization, hypertension which can be severe enough to produce cardiomegaly and heart failure. Changes in retinal blood vessels consistent with hypertension have been seen. This hypertension reflects a defect in steroid metabolism different from that present in the other groups.

### HYPERTENSION AND ADRENOCORTICAL INSUFFICIENCY

In the past few years three adrenogenital infants who have a hitherto undescribed clinical form of the disease have been studied by Bartter and Irons [36] (Fig 20-1). They exhibited in addition to hypertension signs of adrenal insufficiency characterized by sensitivity to salt deprivation. Two of the patients are female pseudohermaphrodites and the third is a brother of one of them.

### CHOLESTEROL BIOSYNTHESIS

Prior to a discussion of the altered steroid synthesis associated with congenital adrenal hyperplasia, the normal pathways of steroid biosynthesis will be described. Cholesterol is the most important, if not indeed the sole precursor of the steroid hormones. Before a discussion of adrenal corticoid biosynthesis can be undertaken, attention must be devoted to the biochemical origins of this key metabolite.

Because of the relatively large size and complexity of cholesterol (the structure was established early in 1912 [37]), the problem of its biosynthesis was very difficult. In its solution a vital role was played by the organic chemists who provided remarkably accurate prediction about routes and mechanisms in the complicated series of reactions leading to the sterols, but it was only through painstaking carbon-by-carbon de-

gradations of isotopically labeled cholesterol and its synthetic intermediates [34-41] that the presently accepted path of biosynthesis was verified



Fig 20-1 Female pseudohermaphrodite 14 months old with virilizing congenital adrenal hyperplasia thought at birth to be a male with hypospadias. At 1 day because of cyanosis, labored breathing and a cardiac arrhythmia she was admitted to a hospital where the diagnosis of congenital adrenal hyperplasia with adrenal insufficiency was made. It is of particular interest that she not only had adrenocortical insufficiency with associated electrolyte disturbances but also was hypertensive with systolic blood pressures of 150 to 180 mm Hg and occasionally higher. Without steroid therapy and added dietary salt, hyperkalemia and hyponatremia appeared rapidly. The elevated blood pressure and adrenal insufficiency are now corrected by the oral administration of 12.5 mg cortisol per day and added salt in her diet. Several months after the photograph a clitoridectomy was performed with a satisfactory cosmetic result. The patient is under the care of P. Pronove and F. C. Bartter of the National Institutes of Health. (Permission of P. Pronove and F. C. Bartter.)

#### IN VIVO SYNTHESIS OF CHOLESTROL FROM ACETATE

For the sake of clarity the biosynthetic reactions will be described as they occur sequentially rather than as they were unraveled historically. First of course it was necessary to establish that mammalian organisms can in fact synthesize cholesterol. Balance studies [42] demonstrated this since the total body cholesterol of the experimental animals remained constant even when they were placed on a cholesterol free diet. The historic isotope experiments of Rittenberg and Schoenheimer [43] confirmed this view and in addition suggested that the sterol is made from small molecules rather than large preformed precursors. Experiments conducted on ergosterol<sup>1</sup> formation in microorganisms [44] suggested that the precursor might be acetate. Another approach made use of a *Neurospora* mutant which requires acetate for growth [45] and led to the same conclusion. When the mold was grown on glucose in the presence of labeled acetate the isotope content of the sterol isolated from the cul-

<sup>1</sup> This plant sterol which differs from cholesterol only by an additional methyl group and double bond in the side chain is sufficiently close structurally so that studies on its biogenesis have been of great help in understanding cholesterol biosynthesis.

ture was the same as that of the isotopic precursor. This finding indicated that at least in this organism acetate is the sole sterol precursor. Many other experiments with mammalian organs, principally liver [46-48], have confirmed these earlier findings. More recently the stepwise degradation of cholesterol biosynthesized from either methyl- or carboxyl-labeled acetate- $C^{14}$  has firmly established what could only be inferred from the previous experiments, namely that each carbon in the sterol molecule arises from either the carboxyl or methyl carbon of acetate [48-51, 49].

Especially relevant to present considerations is the work of Srere et al [50] who showed that the adrenal gland also can convert acetate to cholesterol. Later work by these investigators [51] has demonstrated that in fact almost all mammalian tissues can carry out this biosynthesis.

#### ACETATE ACTIVATION

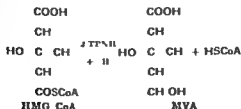
The extensive investigations of Lipmann and Lynen [see 52] and their respective collaborators have established that the free acetate ion ( $CH_3COO^-$ ) does not itself participate in biosynthetic reactions but that it must first be metabolically activated by condensation with the pantothenic acid-containing coenzyme A to form a thiol ester, acetyl coenzyme A. The formation of acetyl CoA has been shown by Berg [53, 54] to occur in two steps: first a reaction of acetate with ATP to form acetyl adenylate (acetyl AMP) followed by transfer of the acetyl moiety to coenzyme as follows:



#### FORMATION OF MEVALONIC ACID

The earliest reaction of acetyl CoA in sterol formation is the condensation of two molecules to form acetoacetyl CoA, which in turn reacts with a third acetyl CoA to yield the CoA derivative of  $\beta$ -hydroxy- $\beta$ -methylglutaric acid (HMG CoA) [55, 56] (Fig. 20-2).

Recently [57] the further metabolism of HMG CoA has been shown to involve its reduction and decarboxylation to produce mevalonic acid (MVA) as follows:





This reaction is noteworthy in several respects. It proceeds without the accumulation of an aldehyde intermediate mevaldic acid. Wright [58] and Lynen [59] have shown however that the latter can be reduced to MVA when introduced into the appropriate system.

In addition the HMG MVA conversion appears to be the rate limiting step in sterol biogenesis which means that physiologic influences affecting the rate of cholesterol synthesis [60] probably act on this reaction.

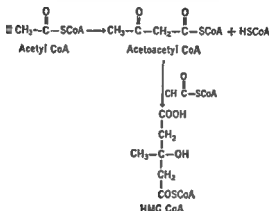


Fig. 20-2 Biosynthesis of HMG CoA

### FORMATION OF SQUALENE

That MVA is an efficient cholesterol precursor was a finding of major importance since at the time it was the only definite link between acetate and the 30-carbon intermediate squalene. MVA originally came to light as the acetate-replacing factor of *Lactobacillus casei*; subsequently it was tested [61] as a sterol antecedent and found to be very effective. Further work in which squalene [62-63] and cholesterol [64] biosynthesized from MVA  $\text{C}^{14}$  were degraded has indicated that following decarboxylation the five remaining carbons of MVA are converted directly to the sterol. The intermediate reactions are presently the subject of intensive investigation. Enzyme studies [65] have shown that mevalonic acid is phosphorylated to phosphomevalonic acid (MVAP) by ATP in the presence of a yeast enzyme mevalonic kinase and that this phosphorylation is a prerequisite to the conversion of MVA to sterol. More recent evidence [66] suggests that a further phosphorylation of MVAP also at the expense of ATP yields MVA pyrophosphate (MVAPP) which can lose water and  $\text{CO}_2$  to form isopentenyl pyrophosphate. The latter isomerizes according to a preliminary report [67] to form  $\gamma\gamma$  dimethylallyl pyrophosphate (Fig. 20-3).

On the basis of indirect evidence Rilling et al. [68] had concluded that the hydrocarbon farnesene ( $\text{C}_{15}\text{H}_{26}$ ) was intermediate between MVA

and squalene. More recently the Lynen group [69] has shown that the intermediates between isopentenol PP and squalene are geranyl PP and farnesyl PP and that 2 moles of farnesyl PP can condense in a reductive

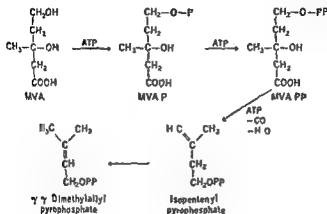


Fig 20-3 Conversion of MVA to  $\gamma\gamma$ -dimethylallyl pyrophosphate

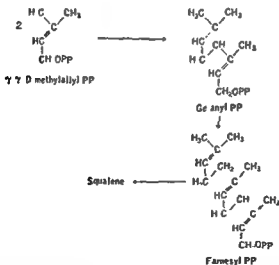


Fig 20-4 Formation of squalene from  $\gamma\gamma$ -dimethylallyl pyrophosphate

reaction to become squalene (Fig 20-4). They have also suggested a general mechanism for the biosynthesis of other terpene derivatives via condensation of isopentenyl pyrophosphate based on the bond energy of pyrophosphoric acid esters and the tendency of these unsaturated compounds to form carbonium ion intermediates.

## CYCLIZATION OF SQUALENE

It is interesting that as early as 1926 it was suggested that squalene was a cholesterol precursor [70-71] because when it was fed to rats their liver and blood cholesterol concentrations increased. Shortly thereafter Sir Robert Robinson [72] suggested a plausible mechanism by which squalene could cyclize to produce a sterol. Nearly 30 years later, this suggestion and a much newer alternate hypothesis advanced by Woodward and Bloch [73] were tested, and the former was rejected. The role of squalene as a sterol precursor, however, has been quite definitely established.

Closure of the squalene molecule which results in the first sterol intermediate, lanosterol [74] (Fig. 20-5) is one of the most complicated

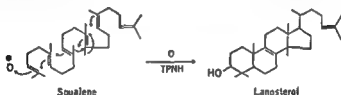


Fig. 20-5 : The cyclization of squalene. Arrows indicate movement of electron pairs.

apparently single step biochemical reactions known. An ingenious mechanism for it has been proposed by Ruzicka [75] in which a cationic oxygen atom attacks what will become carbon number 3 of the steroid nucleus. There follows a series of electron displacements, carbonium ion intermediates, and 1,2 methyl shifts, and a final stabilization of the structure by ejection of a proton. These brilliant speculations have not yet been decisively established, but they are rendered probable by the findings that the entire squalene-lanosterol conversion proceeds without the uptake of hydrogen from the aqueous medium and that the oxygen introduced at carbon 3 is derived from atmospheric oxygen [76].

## CONVERSION TO CHOLESTEROL

The conversion of lanosterol ( $C_{30}$ ) to cholesterol ( $C_{27}$ ) (Fig. 20-6) requires the removal of three extra methyl groups which are eliminated as  $CO_2$  [77], the reduction of two double bonds, one at position 8 and the other in the side chain, and finally unsaturation at carbon 5.

The available evidence [78-80] suggests that first the methyl group at position 14 and then the *gem* dimethyl groups at 4 are removed to produce zymosterol. There follows the formation of desmosterol in which the double bond, previously at 8, has been shifted to position 5. The introduction of the latter unsaturations may proceed by hydroxylation at either 5 or 6 followed by removal of water. Finally, cholesterol is produced by reduction of 24-25 unsaturation.

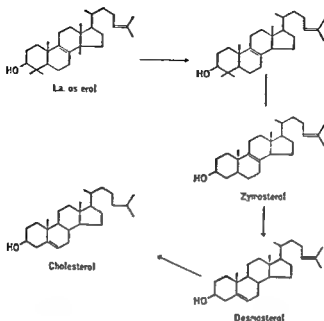


Fig 20-6 The synthesis of cholesterol from lanosterol

## ADRENOCORTICAL STEROID BIOSYNTHESIS FROM CHOLESTEROL

Cholesterol is a precursor of the adrenal steroids under many experimental circumstances. As with cholesterol biogenesis, the insight of the organic chemists preceded experimental verification of the synthetic routes. The first demonstration that cholesterol is a steroid hormone precursor was by Bloch [81] who showed that deuterium labeled cholesterol is converted to labeled pregnanediol. The latter was isolated from the urine of a pregnant woman. The conversion of cholesterol to the corticoids has also been found both *in vivo* [82] and *in vitro* [83].

### PREGNENOLONE FORMATION

The initial attack on the cholesterol molecule results in cleavage of the side chain to yield isocaproic acid and pregnenolone [84-86], a 21 carbon steroid which retains the 3 $\beta$  hydroxyl group of cholesterol and its 5-6 unsaturation (Fig 20-7). The detailed mechanism of conversion is not known. There is evidence to suggest that 20-hydroxycholesterol may be an intermediate. Enzyme studies have shown that the cleavage reaction is in some way dependent on DPN and ATP but the necessity for these coenzymes does not at the moment help in understanding the reaction mechanism.

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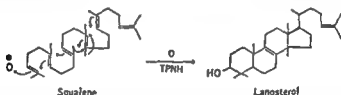


Fig. 20-5. The cyclization of squalene. Arrows indicate movement of electron pairs.

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## CONVERSION TO CHOLESTEROL

The conversion of lanosterol ( $C_{30}$ ) to cholesterol ( $C_{27}$ ) (Fig. 20-6) requires the removal of three extra methyl groups which are eliminated as  $\text{CO}_2$  [77], the reduction of two double bonds, one at position 8 and the other in the side chain, and finally unsaturation at carbon 5.

The available evidence [78-80] suggests that first the methyl group at position 14 and then the *gem* dimethyl groups at 4 are removed to produce zymosterol. There follows the formation of desmosterol in which the double bond previously at 8 has been shifted to position 5. The introduction of the latter unsaturations may proceed by hydroxylation at either 5 or 6 followed by removal of water. Finally, cholesterol is produced by reduction of 24-25 unsaturation.

ensus recently summarized by Hechter [88] seems to be that progesterone is initially hydroxylated at 17 then at 21 and finally at 11. The mechanism of these hydroxylations is not well understood (see below). The enzyme system for 11 hydroxylation is found in the mitochondria [89] at least in bovine adrenal glands while the 17- and 21 hydroxylases are associated with the microsomal fraction [90-91]. Each reaction requires the participation of TPNH.

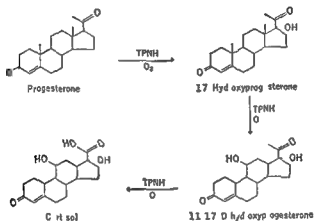


Fig 20-9 Biosynthesis of cortisol from progesterone

#### ALDOSTERONE FORMATION

Although not of principal concern here it is interesting to note that cholesterol can act as a precursor of the salt retaining hormone aldosterone in cell free preparations of mammalian adrenal glands (reported in [88]). In addition to those enzymes mentioned earlier the only enzymatic machinery required for such a reaction is an 18-hydroxylase and a dehydrogenase capable of oxidizing the 18-hydroxyl group to an aldehyde and possibly several enzymes for the formation of the hemiacetals. As with cortisol biosynthesis there is current disagreement about the order of the required hydroxylations when progesterone is the starting material. The available evidence does not indicate a unique biosynthetic pathway from a particular  $C_{21}$  aldosterone precursor but only shows that aldosterone formation is possible from corticosterone, cortisone (11-deoxycorticosterone) or progesterone.

#### BIOSYNTHESIS OF ADRENAL ANDROGENS

The preceding sections have shown how cholesterol is converted into adrenocortical hormones. Perhaps the most remarkable feature of this pathway is that a single reaction, hydroxylation, occurs over and over

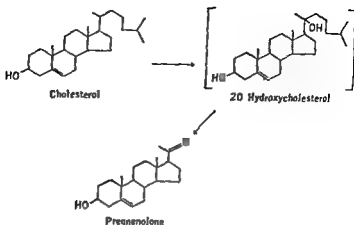


Fig 20-7 : Pregnenolone formation from cholesterol

### OXIDATION TO PROGESTERONE

The next step toward the corticoids is the transformation of pregnenolone into progesterone (Fig 20-8). Although the oxidation was initially assumed to be catalyzed by a single enzyme, a  $3\beta$  hydroxysteroid dehydrogenase [86], the process consists of two distinct chemical changes

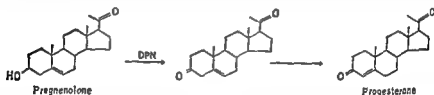


Fig 20-8 Conversion of pregnenolone to progesterone

in pregnenolone (1) oxidation of the hydroxyl group at 3 to form a ketone (2) isomerization from a  $\Delta^5$  to a  $\Delta^4$  unsaturated compound. It seems much more likely, therefore, as suggested by Wang and Talalay [87] that the oxidation of the 3 hydroxyl group is catalyzed by one enzyme and the irreversible migration of the double bond is catalyzed by a second, a steroid isomerase.

### HYDROXYLATION OF PROGESTERONE

Progesterone when compared with the desired end product cortisol (Fig 20-9) lacks three hydroxyl groups  $11\beta$ ,  $17\alpha$  and  $21$ . The order in which these are affixed to progesterone is debatable and it is not clear at present whether there is any required sequence of hydroxylation. A reasonable amount of evidence suggests that progesterone can be hydroxylated initially in any of the three required positions but the con





again as the molecule is altered sequentially. It should come as no surprise then that this same mechanism is important in androgen biosynthesis.

Androgen production from cholesterol [88] and progesterone [92] has been demonstrated in adrenal tissue. Although these conversions have not been studied so extensively as in the testis, it may be assumed that the reactions do not differ significantly in the two organs. In testis the key finding has been that 4 androstenedione can be formed by cleavage of the 2 carbon side chain of  $17\alpha$  hydroxyprogesterone [93-95] (Fig. 20-10). The synthetic sequence leading from progesterone to androgens

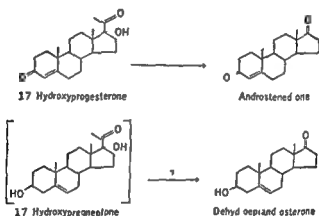


Fig. 20-10 Conversion of a C<sub>21</sub> to a C<sub>19</sub> steroid

appears therefore to be first 17 hydroxylation of progesterone (which has already been discussed) followed by loss of the side chain by what appears to be a hydroxylation. The two carbons eventually appear as free acetic acid.

An unsettled problem concerns the origin of dehydroepiandrosterone (3 $\beta$  hydroxy androst-5-ene-17-one). Because it retains two of the characteristic features of cholesterol (a double bond at 5 and the 3 $\beta$  hydroxyl group) it is apparently not derived from progesterone or 17 hydroxyprogesterone. Two plausible schemes have been advanced to explain its presence. The first, which involves a direct synthesis from cholesterol with removal of the 8-carbon side chain, is not supported by direct evidence. The second, which requires less revision of established pathways, proposes by analogy with the 17 hydroxyprogesterone to androstenedione conversion that dehydroepiandrosterone arises from 17 hydroxypregnenolone. Unfortunately, in his study of the latter, Lynn [96] did not test 17 hydroxypregnenolone as a substrate although the reaction appears probable.

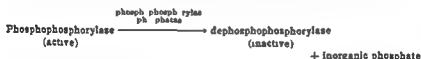
There are small differences two amino acids between the species Secondary structure (e.g.  $\alpha$  helical configuration) appears to be lacking since no evidence of hydrogen bonded interaction has been obtained from deuterium exchange experiments [109]

A remarkable coincidence has appeared as a result of work on the ACTH structure The melanocyte-stimulating hormone (MSH) the structure of which has recently been determined [110-111] shares a sequence of seven amino acids with ACTH Thus it appears that the two peptides physiologically so different must have both genetic and functional characteristics in common Of incidental interest is that the common sequence explains the inherent MSH activity of purified ACTH [112] The structure of ACTH has not been confirmed by synthesis but work has already been carried to the point where peptides with MSH activity have been prepared [113-115]

Before discussing ACTH action it is necessary to examine some aspects of carbohydrate metabolism The investigations of Sutherland and his collaborators [116-118] on phosphorylase have been illuminating This protein which catalyzes the reversible phosphorylation of glycogen



exists in both an active and an inactive form The active molecule in which one of the serine residues is phosphorylated [119] is termed phosphophosphorylase and can be inactivated by a second enzyme phosphophosphorylase phosphatase [117] which hydrolyzes the phosphate from the phosphoserine



Active phosphorylase can be regenerated by phosphorylation of the serine residue with ATP in the presence of still a third enzyme dephosphophosphorylase kinase [118]



This remarkable cycle has important physiologic consequences as concurrent work in Sutherland's laboratory has shown [120] The hormones epinephrine and glucagon which facilitate glycogen breakdown do so by stimulating the phosphorylation of inactive phosphorylase through the kinase reaction thereby activating inactive dephosphophosphorylase Stimulation of the kinase is affected in some way by a

The evidence for an alternate pathway in which cholesterol is not an intermediate is derived from isotope experiments using precursors of both cholesterol and the hormones e.g. acetate. Under some circumstances, after incubation of adrenal tissue with such a labeled compound, the cholesterol isolated at the end of the experiment has had a lower specific activity than the hormones which supposedly were derived from cholesterol. Furthermore Brady [98] using hog testis slices, obtained evidence that androgen biosynthesis from acetate *in vitro* could be stimulated by adding follicle-stimulating hormone (FSH) to the system. Under these conditions there was no increase of acetate incorporation into cholesterol. Heard et al [99] have reported that acetate- $C^{14}$  is converted to various corticoids by hog adrenal homogenates and that the adrenal cholesterol does not become labeled at all. Unfortunately these experiments cannot be interpreted unequivocally to mean that cholesterol is not a precursor. Since cholesterol is not uniformly distributed throughout the cell [100] it is possible that acetate might pass through a very small but metabolically active cholesterol pool on its way to the hormones without labeling the total intracellular cholesterol to a significant degree. Brady's experiments could be explained if the rate-limiting FSH-controlled reaction is between cholesterol and the androgens. An increase in the rate of such a process could then increase the isotope incorporation into the hormone without affecting the labeling of the cholesterol. In a recent review [88] Hechter suggested that a smaller isoprenoid intermediate than squalene, for example a 20-carbon hydrocarbon, could give rise to  $C_{27}$  steroid, and other steroids having fewer carbon atoms than cholesterol. Unfortunately there is too little evidence to decide on the merits of such a hypothesis at the moment.

## CONTROL OF ADRENOCORTICAL FUNCTION

For a number of years the feed back mechanism involving the anterior pituitary and the adrenal cortex has been under intensive study by physiologists and clinicians. It has been known for some time [101-103] that the pituitary elaborates an adrenocorticotrophic hormone (ACTH) which stimulates the adrenal cortex to increase its output of cortical steroids [104]. There is an accompanying fall in adrenal cholesterol and ascorbic acid [105]. 11-Hydroxylated cortical steroids inhibit the output of ACTH by the pituitary. Thus a homeostatic mechanism is present which serves to maintain a constant output of the cortical hormones.

As a result of many brilliant recent studies of the pituitary-adrenal relationship a molecular basis for understanding the physiological event is now available. One of the most striking advances has been the elucidation of the complete amino acid sequence of ACTH from both pig [106] and sheep [103]. Both are polypeptides composed of 39 amino acids.

A possible explanation for the discrepancy may be that after the 18 hydroxylation required for aldosterone biosynthesis there must be an oxidation to form the 18 aldehyde. If this oxidative step were in fact very low it might limit the overall rate of aldosterone synthesis and therefore no matter how much the preceding hydroxylation reactions were enhanced by ACTH the rate of aldosterone formation would go no faster than the rate of the oxidation reaction. Aldosterone production would therefore not be appreciably affected by ACTH but would be influenced by any stimulus which controls the rate of aldehyde formation at C-18.

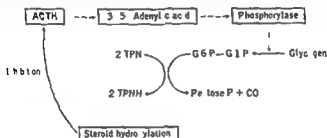


Fig. 20-13 Mechanism of action of ACTH

Another unresolved feature of ACTH action is that it stimulates not only cortical secretion but also growth. In addition to promoting TPNH production, ACTH would provide pentose phosphate since that is a product of the oxidative metabolism of glucose 6 phosphate. It might be that the concentration of pentose phosphate  $\pm$  nucleic acid constituent controls the rate of growth of the adrenal gland. In addition it is quite likely that once the primary action of ACTH is manifest, secondary enzyme changes could occur as a result of enzyme induction, for example, which might result in an increase of adrenal size.

The other limb of the feedback mechanism, the means by which the steroids regulate pituitary ACTH output, is a complete mystery. It appears that pituitary-suppressing activity of adrenal steroids corresponds to their glucocorticoid activity [127].

## THE MECHANISM OF STEROID HYDROXYLATION

The addition of hydroxyl groups to the steroids is of the utmost importance in steroid metabolism. Steroid hydroxylation was first encountered in the squalene oxidoreductase reaction where it was postulated that a positive oxygen ion attacks squalene and initiates the dramatic ring closure in the formation of lanosterol. This oxygen remains with the nucleus to become the  $3\beta$  hydroxyl-oxygen of cholesterol. In the conversion of lanosterol to cholesterol, the three surplus methyl

cyclic nucleotide, 3,5 adenylic acid (Fig 20 12) This synthesis, from ATP in intracellular liver particles [121, 122] is stimulated by epinephrine and glucagon

Although these events have been shown to occur in liver, Haynes [123, 124] has obtained evidence that the situation in the adrenal gland is closely related Here ACTH (but not glucagon or epinephrine) stimulates the synthesis of the nucleotide On the other hand ACTH cannot stimulate synthesis of this nucleotide in liver At any rate once the 3 5

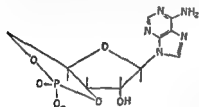


Fig 20-12 3 5 Cyclic adenylic acid

adenylic acid is formed, its apparent function is stimulation of adrenal phosphorylase by the same mechanism as that outlined for the liver

The relation between the activation of phosphorylase by ACTH and increased cortical steroid biosynthesis has been explained by Haynes [123] He argues that since steroid hydroxylation reactions are quantitatively

the most important steps in corticoid biogenesis and since TPNH is required for each hydroxylation the rate of TPNH formation in the adrenal gland could control the rate of steroid hydroxylation and, therefore steroid genesis

How can increase in phosphorylase activity produce a higher TPNH concentration? Under physiologic conditions phosphorylase catalyzes the breakdown of glycogen to yield glucose 1 phosphate which is isomerized to glucose 6 phosphate The latter can traverse one of two metabolic routes the Embden Meyerhof pathway, which results in the formation of pyruvate and DPNH or the oxidative pathway which yields 1 mole of pentose and 2 moles of TPNH per mole of glucose 6 phosphate oxidized It has been demonstrated [125] that the oxidative pathway in adrenal tissue is very active and consequently a large proportion of the glucose 6 phosphate derived from glycogen phosphorolysis will provide TPNH The reduced coenzyme can then be used for steroid hydroxylation [126] and therefore for corticoid androgen and incidentally estrogen biosynthesis This scheme is summarized in Fig 20 13

This sequence of coupled reactions is truly extraordinary The site of ACTH action is so many chemical steps removed from the enzymes directly concerned with production of steroids that a high degree of molecular organization is necessary for this control mechanism to be effective

Satisfying as the over all hypothesis may be several unanswered questions remain The first pertains to the lack of appreciable effect of ACTH on the formation of aldosterone This is surprising since the synthesis of aldosterone which is highly hydroxylated should be affected

react with oxygen to form a metal oxygen complex capable of hydroxylating the substrate

The role of TPNH is likewise not understood. The important studies reported by Kaufman [139] on the conversion of phenylalanine to tyrosine have shown that TPNH is used to reduce a pteridine the function of which is still mysterious. Other reports of additional requirements in steroid hydroxylation [140] may indicate that there too TPNH is only a secondary reducing agent. Tchen and Bloch [141] have suggested that TPNH (or another reducing agent) is used to reduce a metal on the enzyme after its oxidation by reaction with oxygen. Purification and study of the enzymes will undoubtedly yield more enlightening results in the future.

With regard to the question of intermediates one of the earliest hypotheses [142] was that an unsaturated steroid was formed prior to the addition of oxygen but this idea has been quite effectively ruled out. Another interesting suggestion [143] has been that a steroid hydroperoxide is formed which is subsequently reduced by TPNH to the required hydroxy product. Failure to detect such intermediates [144] suggests that this is not the case but the possibility cannot be definitely excluded. The most likely reaction on the basis of studies by Corey and White [138] is direct attack by a positively charged (electrophilic) oxygen on the appropriate carbon atom without formation of a steroid intermediate. The site of the hydroxylation of course would be controlled by a specific enzyme. Preliminary evidence [145] suggests that at least in steroid 11 $\beta$  hydroxylation there are several substrate specific enzymes which determine which of several 11-deoxy substrates 11-deoxy corticosterone (cortexone) or 11-deoxy 17 hydroxycorticosterone (cortexolone) will be hydroxylated.

## BIOCHEMICAL ASPECTS OF THE ADRENOGENITAL SYNDROME

The adrenogenital syndrome can be distinguished from other forms of pseudohermaphroditism and virilism by two features (1) diffuse adrenocortical hyperplasia and (2) large quantities of androgenic 17 keto steroids in the urine. Initially treatment was directed toward these abnormalities. Partial adrenalectomy often resulted in improvement but almost invariably there was progression of the disease as the remaining adrenal cortex hypertrophied. Because of the androgen excess treatment with nonandrogenic 17 ketosteroids and estrogens was tried but without success.

The first advance in therapy was reported by Bartter et al [146] and Wilkins et al [147] in 1950. Wilkins administered cortexone to a female pseudohermaphrodite with virilizing adrenal hyperplasia and found that

groups (two at position 4 and one at 14) are first hydroxylated and then eliminated as carbon dioxide. There is also presumptive evidence that the 5-6 double bond in cholesterol is introduced through hydroxylation of either C 5 or C 6 with subsequent removal of water.

In the series of reactions leading from cholesterol to the cortical hormones, hydroxylation is essential, since removal of the cholesterol side chain probably proceeds by way of at least one hydroxylated intermediate. 20 hydroxycholesterol. Progesterone once formed is converted to cortisol by three additional hydroxylations at 11, 17, and 21. Androgen biosynthesis from 17 hydroxyprogesterone also involves a hydroxylation reaction. Many microorganisms, particularly the fungi [128] have the ability to hydroxylate steroids at almost all positions.

In addition to the steroids many other reagents are hydroxylated. In the following discussion no distinction will be made between aromatic and steroid hydroxylation. Support for a common mechanism in both comes from the fact that various nonenzymatic 'model' systems [129, 130] can promote the hydroxylation of both types of substrates [131, 132].

Several features characterize all the reactions studied thus far. The first (shown by the use of isotopic oxygen e.g. [134]) is that the oxygen which becomes bound to the substrate is derived from atmospheric oxygen and not from the aqueous environment. Hydroxylation is therefore an oxidation in the classic sense. The second common finding is that a reducing agent, usually TPNH is required. In a few cases [134, 135] other reducing agents have been active.

Enzymes which catalyze reactions of this type have been classified by Mason [130] as "mixed function oxidases". The over all reaction can be generalized as follows:



where  $XH$  = the substrate e.g. steroid to be hydroxylated.

The biochemical and chemical aspects of this problem which have been investigated are (1) What sort of active oxygen is used? (2) At what stage is the reducing agent used? (3) Is the substrate 'activated' and do any intermediates involving the substrate occur?

With regard to the first of these the consensus based on experiments with model hydroxylating systems [129] and on Ruzicka's [75] speculations is that cationic oxygen ( $O^+$ ) is the attacking reagent. Recent results obtained by Bartlett [137] have suggested the way in which such an intermediate can be produced chemically and a series of biochemical and chemical experiments performed by Corey and various associates [138] has illustrated how such a reactant could attack steroids.

The means by which reactive oxygen is produced biochemically is unknown. Metal containing enzymes—possibly hemoproteins [130]—may

Pregnane-3 $\alpha$  ol 11 20 dione

Pregnane 3 $\alpha$  17 $\alpha$  diol 20-one<sup>[8]</sup> (17 hydroxypregnanolone)

Pregnane 3 $\alpha$  17 $\alpha$  20 $\alpha$  triol 11 one (pregnanetriolone)<sup>[5]</sup>

Pregnane 3 $\alpha$  11 $\beta$  17 $\alpha$  20 $\alpha$  tetrol<sup>[1]</sup>

Pregnane-3 $\alpha$  ol 20-one

In addition many urinary 17 ketosteroids are increased [173 177 179 180 183 184 187]

Androsterone<sup>[13]</sup>

Etiocholanolone<sup>[13]</sup>

11 Ketoetiocholanolone<sup>[11]</sup>

11 $\beta$ -Hydroxyandrosterone<sup>[12]</sup>

11 Ketoandrosterone<sup>[11]</sup>

11 $\beta$  Hydroxyetiocholanolone<sup>[7]</sup> has been reported both increased [187] and absent [180] The ratio of 11 oxygenated 17 ketosteroids to total 17 ketosteroids is consistently increased

The possibility of 21 deoxycorticoids resulting from the 21 dehydroxylation of hydrocortisone rather than from a primary defect in 21 hydroxylation has been considered and ruled out by Fukushima and Gallagher [188] C<sup>14</sup> Cortisol was administered to a patient with the adrenogenital syndrome and less than 0.5 per cent of the radioactivity was recovered in 21 deoxysteroids

#### *Pregnanetriol and Pregnanetriolone*

Excessive excretion of pregnanetriol<sup>[6]</sup> in the adrenogenital syndrome was first reported in 1937 by Butler and Marrian who noted its disappearance following partial adrenalectomy in one patient with this disease [189] It was also reported in the urine of virilized adults by others [183 184] and a simple test for it was devised in 1953 by Bongiovanni who then surveyed children with the adrenogenital syndrome and found increased amounts regularly present in their urine [190 191] Since pregnanetriol is the major corticosteroid metabolite in the urine of untreated patients it has become a valuable aid in establishing the diagnosis of the adrenogenital syndrome [168]

11 $\beta$  Pregnanetriolone<sup>[5]</sup> was first isolated from the urine of patients with the adrenogenital syndrome by Finkelstein and associates [186] As they [186 192] have pointed out the presence of this steroid in the urine may be of greater diagnostic significance than that of pregnanetriol It is always present in increased quantities in congenital adrenal hyperplasia and unlike pregnanetriol has been absent in the cases of adrenal carcinoma thus far examined

Treatment with cortisone or any of a variety of steroids which presumably inhibit the pituitary gland results in a prompt decline in urinary pregnanetriol and 17 keto steroids [168 190]



urinary 17 ketosteroids which averaged 48 mg per day prior to treatment fell on the second day, and after the ninth day averaged 6.5 mg per day. No change in the virilism was observed in the 15-day treatment period. Since then there have been numerous reports of treatment with various corticosteroids [8, 11, 12, 35, 148-164] which have suppressed the signs of the adrenogenital syndrome and permitted growth and sexual maturation and in the female ovulation and pregnancy.

Bartter et al. [146, 148] found that ACTH stimulated the production of 17 ketosteroids in these patients but, as had also been reported by Lewis and Wilkins in 1949 [160], did not produce an immediate  $K^+$  loss or retention of  $Na^+$  and  $Cl^-$  in contrast to what occurs in normal individuals. Cortisone administration on the other hand caused a decrease in 17 ketosteroids and was found to affect salt balance as in normal individuals but produced a more pronounced nitrogen loss.

The following outline, which has been of great value in understanding the disease, was then proposed by Bartter:

1. The primary defect is the decreased production of the 'sugar' hormone by the adrenal cortex.
2. There is increased ACTH production because of failure of inhibition of ACTH secretion by 'sugar' hormones.
3. Androgen production is increased.

#### DEFECTIVE HYDROXYLATION AT C-21

A more specific suggestion by Jailer in 1953 was that the hydroxylation of 17 hydroxyprogesterone at 11 and 21 to form hydrocortisone was impaired [160]. In confirmation of these hypotheses, all evidence now indicates that in the most common form of the syndrome there is at least a partial block in the hydroxylation of C-21.

Blood 21 hydroxycorticosteroids [104, 167-172] and urinary metabolites of hydrocortisone are decreased [173]. If these potent inhibitors of ACTH secretion are diminished, one would expect an increase in plasma ACTH. This indeed was observed in 1953 by Sydnor and associates [174]. The adrenal gland is therefore stimulated by excessive ACTH to produce large quantities of steroids which cannot be hydroxylated at C-21.

The following urinary steroids, none of which is hydroxylated at C-21, have been detected [168, 173, 176-186] in increased quantities in the adrenogenital syndrome:<sup>2</sup>

Pregnane-3 $\alpha$ , 17 $\alpha$ , 20 $\alpha$  triol (pregnanetriol) [10]

Allopregnane-3 $\alpha$ , 17 $\alpha$ , 20 $\alpha$  triol [10]

Pregnane-3 $\alpha$ , 20 $\alpha$  diol (pregnanediol) [4]

<sup>2</sup> In the following discussion of steroid metabolism the numbers in squares and superscript letters following names of steroids refer to Fig. 20-14 where their structure and relationships are illustrated.

The presence of the unusual urinary steroids can be largely explained by a defect in 21 hydroxylation the consequences of which are shown in Fig 20-14 The unusual steroids are products of alternate routes of metabolism of compounds whose major route is blocked in this syndrome For example the administration of  $17\alpha$  hydroxyprogesterone[5] to patients with this syndrome results in increased excretion of pregnanetriol[10] and 17 ketosteroids [168] It is likely that part of the increased 17 ketosteroids excreted in this syndrome is derived from  $17\alpha$  hydroxyprogesterone as shown in this figure Fukushima et al have demonstrated the conversion of deuterated  $17\alpha$  hydroxyprogesterone[9] to etiocholanolone[11] and androsterone[12] [193] and Jailer was able to simulate the urinary steroid pattern of congenital adrenal hyperplasia by the administration of  $17\alpha$ -hydroxyprogesterone[3] and 21 deoxycortisol[7] [194] Burstein et al [195] have reported the excretion of  $3\alpha$   $17\alpha$  dihydroxy pregnane 11 20-dione[8]  $11\beta$ -hydroxyetiocholanolone[12] and etiocholane  $3\alpha$  ol 11 17 dione[11] following the administration of 21 deoxycortisone[7] to a patient with rheumatoid arthritis Fukushima et al [196] have recently examined the radioactive metabolites following the administration of 21 deoxycortisol-4  $C^{14}$  to a normal subject and an untreated patient with congenital adrenal hyperplasia and found them to be similar This similarity indicates that the appearance of compounds such as pregnanetriolone[5] in the urine of individuals with the adrenogenital syndrome reflects the large quantities of 21 deoxycortisol[7] which

Fig 20-14 Pathways of corticoid synthesis in the adrenogenital syndrome Asterisks indicate urinary steroids that are abnormal or increased Metabolites of 1-deoxycortisol [196] are marked with daggers and their arrangement is in part arbitrary e.g. compound 11 could arise from compound 7 via 5 6 or 19

- †1 5 $\beta$ -pregnane-3 $\alpha$  11 $\beta$  17 $\alpha$   $^{20}O\alpha$  tetrol
- †1a 5 $\beta$  pregnane 3 $\alpha$  11 $\beta$  1  $^{20}O\beta$  tetrol
- †2 3 $\alpha$  11 $\beta$  17 $\alpha$ -trihydroxy 5 $\beta$  pregnan  $^{20}$ -one
- 3 pregn-4-ene-3  $^{20}$ -dione (progesterone)
- 4 5 $\beta$ -pregnane 3 $\alpha$   $^{20}\alpha$  diol (pregnanediol)
- †5 3 $\alpha$  17 $\alpha$   $O\alpha$ -trihydroxy 5 $\beta$ -pregnan 11-one (pregnanetriolone)
- †5a 3 $\alpha$  17 $\alpha$  20 $\beta$  trihydroxy 5 $\beta$  pregnan 11-one
- †6 3 $\alpha$  1  $\alpha$ -dihydroxy 5 $\beta$  pregnan 11  $^{20}$ -d one
- 7 11 $\beta$  17 $\alpha$ -dihydroxypregn-4-ene-3  $^{20}$ -dione (21-deoxycortisol)
- 8 1 $\alpha$ -1 hydroxypregn-4-ene-3  $^{20}$ -d one (11 $\alpha$  1 hydr typrog t ron )
- 9 3 $\alpha$  17 $\alpha$ -dihydroxy 5 $\beta$ -pregnan  $^{20}$ -one
- 10 5 $\beta$  pregnane-3 $\alpha$  17 $\alpha$  20 $\alpha$ -triol (pregnanetriol)
- 10 5 $\alpha$ -pregnane-3 $\alpha$  1 $\alpha$   $^{20}\alpha$ -triol
- †11 3 $\alpha$ -hydroxy-5 $\beta$  androstan 11  $^{11}$ -dione
- †11a 3 $\alpha$ -hydroxy-5 $\alpha$ -androstan 11 1 $\alpha$ -dione
- †1 3 $\alpha$  11 $\beta$ -dihydroxy-5 $\beta$ -androstan 1 $\alpha$ -one
- †1 3 $\alpha$  11 $\beta$ -dihydroxy 5 $\alpha$ -andro-tan 1 $\alpha$ -o e
- 11 3 $\alpha$ -hydroxy-5 $\beta$ -androstan 1-one (etiocholanolone)
- 13a 3 $\alpha$ -hydroxy-5 $\alpha$ -andro tan 17-one (andro t rone)
- 14 17 $\alpha$  11-d hydroxypregn-4-ene-3  $^{20}$ -d one (compound S)
- 15 11 $\beta$  1 $\alpha$   $^{21}$  trihydroxypregn-4-ene-3  $^{20}$ -d one (compound F cortisol)
- 16 3 $\alpha$  11 $\beta$  17 $\alpha$  11 trihydroxy-5 $\beta$ -pregnan  $O$ -one (tetrahydrocortisol THF)

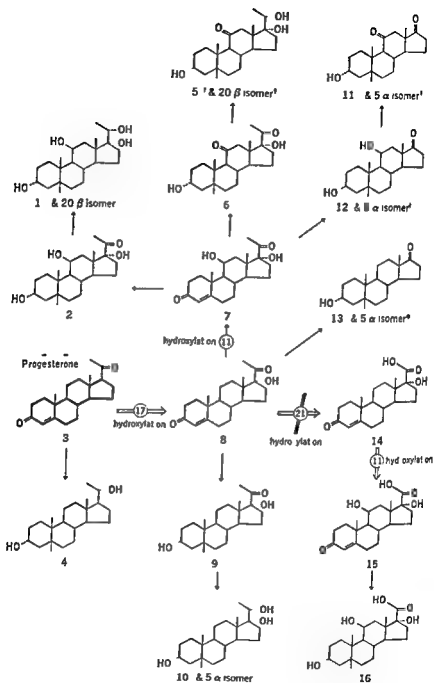


Fig 20 14 For descriptive legend see opposite page

These *in vitro* examinations of adrenal glands from two individuals with the adrenogenital syndrome support the concept of defective 21 hydroxylation and unimpaired 11 hydroxylation in this disease

#### SALT LOSING FORM OF THE ADRENOGENITAL SYNDROME

In the group of patients characterized as salt losers no tetra hydrocortisol<sup>[16]</sup> is excreted and there is as a rule even more urinary pregnanetriol than in the previous group. This indicates that the block in 21 hydroxylation is more nearly complete. The adrenal gland even though overstimulated by ACTH is unable to produce enough 21 hydroxylated steroids to prevent salt loss and signs of adrenocortical insufficiency. There is an intermediate group of patients who are able to produce reduced but apparently adequate amounts of hydrocortisone and who became salt losers only in the presence of superimposed stress such as infection [32].

An alternative explanation for the loss of salt is that there is a salt diuretic hormone produced by the overstimulated adrenal gland. This speculation is due primarily to the observation that ACTH administration may cause transient salt loss in this group of patients [29]. Also Prader et al. reported that aldosterone excretion was normal or elevated in six patients and concluded that salt loss is due to a sodium diuretic factor which in some patients is offset by increased aldosterone production [199]. On the other hand Blizzard et al. [200] have found that aldosterone is low in these patients even in the presence of sodium depletion.

Recently two observations have supported the proposal that these patients produce a salt-excreting factor. Klein and associates [201] have described a substance obtained by paper chromatography of urine from the following sources: premature infants following ACTH administration; infants with congenital adrenal hyperplasia with and without adrenocortical insufficiency; and one patient with congenital adrenal hyperplasia being treated with cortisone. This material when injected into intact white rats caused excretion of sodium. This did not occur with several known steroids including SC 5233 (see below). In addition Neher et al. [202] have isolated two urinary steroids:  $3\alpha$  16 $\alpha$ -dihydroxy pregnan-20-one and  $3\alpha$  16 $\alpha$ -dihydroxyallopregnan-20-one from newborn infants, a pregnant woman, a patient with Cushing's syndrome and three patients with the salt losing form of the adrenogenital syndrome. The first of these steroids is reported to cause salt loss in adrenalectomized male rats but data regarding this effect have not yet appeared.<sup>\*</sup> Although

<sup>\*</sup> Neher et al. *Helvet. chim. acta* 41: 1667, 1958 have found that  $3\alpha$  16 $\alpha$ -dihydroxyallopregnane-20-one isolated from hog adrenal promotes sodium loss in a renal ectomized rat. Cojpage and Liddle *J. Clin. Endocrinol.* 20: 779, 1960 could not demonstrate this effect in human subjects who received up to 400 mg per day of the steroid.

accumulate "behind the block in 21 hydroxylation Pregnanetriolone<sup>[1]</sup> was found to be the chief metabolite of 21 deoxycortisol That 11 oxygenated 17-ketosteroids were found to represent less than 1 per cent of recovered steroid radioactivity was interpreted to mean that the large amounts usually found in the adrenogenital syndrome are mainly derived from C<sub>19</sub> precursors 17 Hydroxyprogesterone itself is probably only weakly androgenic [197] and the virilization characteristic of the disease is undoubtedly due to C<sub>19</sub> steroids such as androsterone which can be derived from 17 hydroxyprogesterone

Barter suggested that although the adrenal gland appeared to be "insensitive" to ACTH, it might be stimulated by the increased secretion of ACTH to produce adequate "sex hormone" [146, 148] This in fact seems to be the case for most of the patients do not have adrenal insufficiency Their plasma contains 17 hydroxycorticosteroids and normal amounts of tetrahydrocortisone are excreted by some [53] Further evidence that 21 hydroxylation is not totally impaired is the increase in blood and urine corticoids in some of these patients following ACTH administration [168]

In view of the enormous amounts of pregnanetriol and 17 ketosteroids excreted relative to decreased or normal amounts of tetrahydrocortisone one is led to the concept of an impaired conversion of 17 $\alpha$ -hydroxyprogesterone<sup>[2]</sup> to hydrocortisone<sup>[15]</sup> In normal individuals this conversion is apparently quite efficient and only minute amounts of pregnanetriol<sup>[1]</sup> are excreted [191]

More direct evidence for the defect in 21 hydroxylation has been obtained by Bongiovanni [196] who examined adrenal glands from two individuals with the adrenogenital syndrome (one of whom had the salt losing form) These adrenal glands were found to contain neither of two 21 hydroxylated steroids compound S (17 hydroxy 11 deoxycorticosterone)<sup>[19]</sup>, and cortisol<sup>[15]</sup> In sharp contrast two normal adrenal glands contained 4 to 6  $\mu$ g per gm of tissue of each of these steroids Present in the diseased adrenal glands were large amounts of 17 $\alpha$  hydroxyprogesterone<sup>[2]</sup> (118 and 155  $\mu$ g per gm of tissue) compared with the amounts in normal adrenal glands (0 and 4.5  $\mu$ g per gm) Homogenates of the diseased and normal glands were tested for their ability to hydroxylate 17-hydroxyprogesterone at C-21 Homogenates of two normal adrenal glands converted 10 per cent and 37 per cent of this substrate to 21 hydroxylated steroids (compounds S and F) Neither of these 21 hydroxylated products was formed in similarly treated homogenates of adrenal glands from two individuals with the adrenogenital syndrome When compared with homogenates of normal adrenal glands the ability of homogenates from two diseased glands to 11 hydroxylate 11 deoxycortisol (compound S)<sup>[19]</sup> to cortisol<sup>[15]</sup> was not impaired and perhaps increased



these observations do not yet permit a conclusion regarding the presence of a sodium-excreting steroid in these patients there is other experimental evidence for such a steroidal effect

Progesterone has been found to inhibit the salt retaining effects of deoxycorticosterone (DOC) in Addison's disease [203] A similar inhibition has been demonstrated in adrenalectomized rats [204] Cella and Kagawa [205] have synthesized 3 (3-oxo-17 $\beta$ -hydroxy-4 androsten-17 $\alpha$  ol) propionic acid  $\gamma$  lactone (SC 5233) and its 19-nor analogue (SC-8109) and have shown them to be more active in this regard than progesterone by factors of 8 and 27 Kagawa and associates [206] and Iiddle [207] have shown that these two synthetic compounds can inhibit the action of aldosterone in rat and man respectively These observations indicate that salt metabolism can be compromised by a progesterone like compound Further experiments will determine the possible role of such a relationship in the salt losing form of the adrenogenital syndrome

### THE HYPERTENSIVE FORM

The defect in steroid metabolism associated with the hypertensive form of the adrenogenital syndrome is apparently different from that in the major syndrome This is inferred from the findings of Eberlein and Bongiovanni [208-210] who studied an 8 year-old female pseudohermaphrodite with congenital adrenal hyperplasia and hypertension

The most striking finding<sup>1</sup> was the presence of large amounts of Reichstein's compound S[9] and tetrahydro S[11] in the blood In addition tetrahydro S was the predominant urinary steroid These compounds produce Porter Silber chromogens which are characteristic of 17 hydroxycorticoids and which are usually low in the syndrome Because the Porter Silber chromogens were not low there was some initial confusion prior to their identification as 11 deoxysteroids The 17 ketosteroids present in the urine were dehydroepiandrosterone androsterone[8] and etiocholanolone[3] In contrast to the findings in the major and salt losing forms of the syndrome no 11-oxygenated 17 ketosteroids were detected and there was a small amount of pregnanetriol[5] excreted relative to the 17 ketosteroids

It is seen in Fig 20-15 that the observed pattern of steroid metabolites in this form of the disease could be explained by a defect in the  $\beta$  hydroxylation of C-11 Tetrahydro S[11] is the reduced form of compound S[9] which ordinarily is hydroxylated at C-11 to form cortisol[13] Pregnan-3 $\alpha$  21 diol 20 one ( tetrahydro DOC)[4] which was also isolated from the urine of this patient is a metabolite of 11 deoxycorticosterone (DOC)[3] It is generally thought that deoxycorticosterone is responsible for the hypertension that characterizes this form of the syndrome

Numbers in squares and superscript letters following steroids refer to corresponding steroids in Fig 20-15

except for elevated urinary 17 ketosteroids have not yet been reported and defects in 11 and 21 hydroxylation can be inferred only by analogy to those children with either the salt-losing or the hypertensive form of the disease

It is germane to this account that there exists in women a fairly well defined syndrome which has been termed an intermediate form of the adrenogenital syndrome. In 1953 Jones et al [21] found that ovulation followed cortisone treatment in a number of women who had slightly elevated urinary 17 ketosteroids and anovulatory cycles. Similar observations have been reported by others [161 213]. In 1958 studies on 6 women with oligomenorrhea or amenorrhea anovulatory temperature curves high normal to elevated urinary 17 ketosteroids and urinary 'pregnane complex' were reported by Gold and Frank [24]. The pregnane complex included pregnanetriol and pregnanediol. Treatment with  $\Delta^1$  cortisone and  $\Delta^1$  cortisol was followed by menstruation ovulatory temperature cycles and depression of 17 ketosteroids and pregnane complex in five patients. Two of these five became pregnant shortly after the steroid treatment began.

The Stein Leventhal syndrome is characterized by oligomenorrhea or amenorrhea hirsutism infertility and bilateral polycystic ovaries. Ovulation usually ensues following wedge resection of the ovaries. A small number of these patients who do not respond to this procedure respond to cortisone therapy by resuming cyclic ovulatory menses.

These individuals perhaps also belong to the intermediate adrenogenital syndrome. Of the six patients reported by Gold four definitely had enlarged polycystic ovaries. This is also of interest since polycystic ovaries occasionally occur in the adrenogenital syndrome. Until there is more information regarding steroid metabolism in these patients the relation of these endocrine disturbances to the adrenogenital syndrome must remain conjectural.

## GENETICS

The familial nature of the adrenogenital syndrome has long been recognized [214]. Knudson [215] reported 11 cases in 8 families in 1951. In a review of the cases reported from 1852 to 1952 Bentinck found 100 cases in 43 families [216]. Childs reported 76 affected children from a total of 181 in 56 families [217]. As pointed out by these authors the distribution is consistent with an autosomal recessive gene which when homozygous produces the syndrome. The fact that the disease affects a single generation is also consistent with a homozygous recessive character. It has also been reported in identical twins [218 19].

One possible inconsistency is the great predominance of affected females. Of the 100 cases in Bentinck's series 83 were probably female.



## VARIATIONS AND UNEXPLAINED OBSERVATIONS

In the preceding discussion the major form of the adrenogenital syndrome has been associated with a defect in 21 hydroxylation and the hypertensive form has been linked with a defect in 11 $\beta$ -hydroxylation. There is probably more variability in the degree and type of defect than was implied in the preceding remarks. For instance pregnane-3 $\alpha$  17 $\alpha$  21 triol 20-one (tetrahydro S) has been detected in the urine of four adrenogenital individuals with the major syndrome without associated hypertension [182, 211]. This steroid which lacks a hydroxyl at C-11 and is hydroxylated at C-21 would certainly not be expected to result from a defect in 21 hydroxylation, the proposed error in the major syndrome.

Dyrenfurth et al. [212] have also described tetrahydro S in the urine of a normotensive female pseudohermaphrodite following ACTH administration. A brother of this patient had the hypertensive form of the disease and also excreted compound S and tetrahydro S. As has been shown, it is not unusual for individuals with the major form of the adrenogenital syndrome to produce some 21 hydroxylated steroids, but their inability to 11 $\beta$  hydroxylate compound S [2] to form cortisol [3] is unexpected. It is of particular interest that two patients described by Birko et al. [211] excreted 11 $\beta$  hydroxyandrostosterone in spite of impairment of the 11 $\beta$  hydroxylation of C<sub>21</sub> steroids indicated by the excretion of tetrahydro S [2] (Fig. 20-15). One of the patients described by Bergstrand et al. [182] excreted not only tetrahydro S but also tetrahydrocortisone; the other patient excreted 11 ketopregnanetriol in addition to tetrahydro S.

There is now additional evidence that obscures the individual nature of the different forms of the syndrome. Bartter and Pronove [36] have studied three infants with the adrenogenital syndrome associated with both adrenal insufficiency and hypertension. If the salt losing form and the hypertensive form each represent independently inherited defects, then their coexistence in a single individual would be extremely rare. That two of the infants with salt loss and hypertension are siblings makes the coincidental occurrence of the two defects in one person even less likely. A more satisfactory explanation would be a single genetically determined defect resulting in the absence of a cofactor or enzyme common to both 21- and 11 hydroxylation.

There are several possible sites for a defect in 11 $\beta$  hydroxylation, since three separate enzymes and a heat stable cofactor, in addition to TPNH and oxygen, are required [140]. A comparable situation may exist with 21 hydroxylation, but the enzyme(s) is less well defined. Adrenal microsomes (from which no active soluble fraction has yet been prepared) TPNH and oxygen are necessary for the reaction [91].

That there is a combination of defects in hydroxylation in these children with the mixed syndrome is only speculative, since steroid metabolites,

■ 21 carbon steroid which following oxidation at C-3 and isomerization from a 5 to a 4 unsaturated compound ■ hydroxylated at 17 21 and 11 to form cortisol

3 Androgenic 19-carbon steroids most probably arise from C<sub>21</sub> steroids by removal of the two-carbon side chain at C-17

4 TPNH is required for several different reactions in steroid synthesis and ACTH apparently stimulates this synthesis through TPNH ACTH causes increased production of 3 5 adenylic acid which stimulates a step in the production of glucose 1 phosphate from glycogen Glucose 1 phosphate is isomerized to glucose 6-P which ■ oxidized to pentose with the production of 2 moles of TPNH

■ There are several clinical forms of the adrenogenital syndrome Each probably represents a variation in the location and degree of hydroxylation defect at C 11 or C 21

The defect at C 21 is most common and results in decreased 21 hydroxylated steroids The pituitary gland uninhibited by these steroids releases increased quantities of ACTH which stimulates the adrenal gland to synthesize excessive 21 deoxysteroids some of which are androgenic or converted to androgens It ■ these androgenic steroids that are responsible for the virilization of these children

6 When the defect in hydroxylation at C-21 is severe, failure of synthesis of C 21 hydroxysteroids may be virtually complete and the deficiency so great that renal salt retention is seriously impaired Infants with such defects are subject to severe salt wasting and may develop Addisonian like crises

7 When the defect involves primarily hydroxylation of C 11 one of the steroid metabolites present in increased amounts is a derivative of 11 deoxycorticosterone Children with a defect in C-11 hydroxylation may show a hypertensive form of the adrenogenital syndrome Presumably the hypertension is attributable to excessive production of 11 deoxycorticosterone

8 The pattern of inheritance of congenital adrenal hyperplasia is most satisfactorily explained by an autosomal recessive gene the several hydroxylation defects being separately inherited There are however inconsistencies in this explanation

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pseudohermaphrodites as were 53 of the 76 individuals reported by Childs. One explanation for this inequality is that females because of the striking genital malformations are more easily detected. This has been supported by Childs finding similar numbers of affected males (9) and females (11) in families in which an individual already had been recognized as having the adrenogenital syndrome. It has also been suggested that there is a higher prenatal mortality in males with the disease but total pregnancy losses of mothers of these individuals are not abnormal.

Since the metabolic defects differ in the hypertensive and major forms of the syndrome it is not surprising that they appear to be inherited separately. In a survey of 18 families containing at least 2 affected children both forms of the syndrome were not observed in a single family [217]. However in a family reported by Birke et al [211] 1 child had the adrenal insufficiency and 2 the major form of the syndrome. Dyrenfurth has reported 2 children with the adrenogenital syndrome the brother having the hypertensive variant and the sister the major form [212].

If the clinical appearance of the adrenogenital syndrome represents the homozygous state of the genetic defect then the parents of affected individuals must be heterozygous. Childs found that the mean increment in pregnanetriol excretion was higher following the administration of ACTH to 20 parents of children with the adrenogenital syndrome than to 18 controls [217].

Examination of the urine of parents of a child with the adrenogenital syndrome revealed high normal 17 ketosteroids and high dehydroisoandrosterone in the mother and low normal 17 ketosteroids and low dehydroisoandrosterone in the father [220]. The parents and three normal siblings of three children with the adrenogenital syndrome were found to have normal excretion of 17 ketosteroids and 17 hydroxycorticosteroids [211].

## SUMMARY

1 Congenital adrenal hyperplasia causes progressive virilization and in girls pseudohermaphroditism. This syndrome results from defects in the biosynthesis of adrenocorticoids.

2 Adrenocorticoids arise from cholesterol, which is synthesized from acetate. The main compounds on the way to cholesterol from acetate are  $\beta$  hydroxy  $\beta$  methyl glutaric acid, mevalonic acid, isopentenyl pyrophosphate, geranyl pyrophosphate and farnesyl pyrophosphate. Two molecules of the latter condense to form squalene which then cyclizes to form lanosterol which is converted to cholesterol. Most of the side chain of cholesterol a 27 carbon steroid is removed to form pregnenolone.

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**Part Six**

**Diseases of Purine and  
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## Chapter 21

### Gout\*

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#### HISTORICAL CONSIDERATIONS

Gout is a disease rich in historical lore. Its descriptions can be traced to the dawn of medical literature. Ancient Greek and Roman physicians possessed an intimate knowledge of its varied features. Over the centuries the differentiation of gout from other articular disorders became clouded and it remained for Thomas Sydenham (1633) to reemphasize this distinction [1]. His masterly description of the disease based in part upon his 34 years of personal affliction marks the beginning of the modern clinical history of gout.

The chemical history of gout began a century after Sydenham when in 1766 Scheele [2] discovered uric acid as a constituent of a kidney stone. Shortly thereafter Wollaston (1797) and Pearson (1798) demonstrated urates in the tophi of patients with gout [3, 4]. Another 50 years later A. B. Garrod performed his historic experiment in which he demonstrated first by the murexide test [5] and later by his famous "thread test" (1854) [6] an increased amount of uric acid in the blood of gouty subjects.

At this time the structure of uric acid was unknown. With the establishment by Emil Fischer [7] in 1893 that uric acid was a purine compound its potential relationship to the nucleic acid constituents adenine and guanine was appreciated and a key role of purine metabolism in the pathophysiology of gout was recognized. With the introduction of a reliable method for determination of uric acid in blood by Folin and Denis in 1913 [8] clinical and metabolic studies in gout were greatly facilitated. A valuable introduction to the history of gout is that of Hartung [9].

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Table 21.1 are cited five representative studies employing three types of methods now in common use. Colorimetric methods such as those of Fohn [16] or Benedict [17] are affected by loss of urate in the protein precipitation step by nonspecificity of color production and by inhibitory and augmentatory influences. These opposing errors cancel each other to a large extent for routine diagnostic use; these or similar methods are quite satisfactory. The upper limit of normal ( $M + 2\text{ s.d.}$ ) for males is about 6.7 mg per 100 ml [12] for females about 5.8 mg per 100 ml.

In order to obviate errors of nonspecificity, colorimetric analyses for uric acid may be performed before and after incubation of the sample with uricase. The values obtained by this procedure are regarded as true uric acid. On an unrestricted diet the upper limits of normal are 7.8 and 5.9 mg per 100 ml in males and females respectively [13].

Currently the preferred method is the direct enzymatic differential spectrophotometric method of Kalekar [18] as modified by Iacobucci [19]. This method involves measurement of the change in absorbance at 292 m $\mu$  of a diluted sample of serum when treated with purified uricase at pH 9.4. The method is sensitive, accurate and specific and it avoids errors inherent in colorimetric methods requiring precipitation of proteins and involving some degree of nonspecificity in color development. By this procedure normal values (2 s.d. level of significance) extend to 7.5 and 5.8 mg per 100 ml for males and females respectively [14, 15].

The sex difference in urate levels is found with all methods but varies in magnitude in different studies. The mean male to female ratio in 10 published studies employing a variety of analytic methods is 1.10 [13, 20]. In children there is normally no sex difference in serum urate levels which are lower than in adults. After puberty the levels rise in both sexes but more so in males so that there is thereafter a higher mean level in males than in females. These differences have been attributed to higher renal clearances of urate in children than in adults and among adults in women than in men [21, 22]. Possible differences of urate production have not been assessed. Finally there is a significant correlation between serum uric acid level and surface area in normal men ( $r = 0.427$ ,  $p = 0.001$ ) but not in normal women ( $r = 0.187$ ,  $p > 0.1$ ) [13]. Smyth, Stecher and Wolfson [23] found that urate levels rose in normal women after the menopause to equal those of men but Hauge and Harvald [15] found the sex difference in their control subjects was lifelong. The mean value was only slightly lower in women under 50 years than in those over this age.

Despite the obvious difficulties of defining valid precise limits to the normal range of serum uric acid value, clinical experience has led physicians to view any value found by a colorimetric method in excess of



Many authors have compiled rosters of distinguished personages in history who have had gout. The list is impressive and includes persons of such diverse claims to fame as the Medici, Isaac Newton, Charles Darwin, Martin Luther, John Calvin, Benjamin Franklin, Ben Jonson, Samuel Johnson, and Goethe [10].

## DEFINITIONS

Primary gout is a genetically determined disorder of purine metabolism in which the cardinal feature is hyperuricemia. The hyperuricemia is initially asymptomatic and may remain so, but in a small percentage of affected individuals the disorder is complicated by episodes of acute arthritis of unknown cause, by chronic arthritis due to depositions of urate in cartilage and other tissues, by formation of renal calculi composed of uric acid, and possibly by an accelerated development of vascular and renal disease.

Asymptomatic hyperuricemia is considerably more common than overt gout. Most students of this disease currently regard asymptomatic hyperuricemia as synonymous with the gouty diathesis and tacitly assume the potentiality for symptomatic gout in each hyperuricemic individual.

A large section of this chapter is devoted to an inquiry into the cause or causes of hyperuricemia in primary gout. Although recent studies have greatly increased understanding of the general nature of one metabolic derangement present in certain patients with gout, the precise biochemical defect or defects leading to hyperuricemia in subjects with this disease is still unknown. Furthermore, even if all factors responsible for hyperuricemia were known, this knowledge would not in itself constitute a full explanation for the disease, for it would not adequately explain the local tissue factors leading to precipitation of urate, nor would it necessarily explain the mechanisms operating to initiate the acute attack of gouty arthritis in a person genetically endowed with the gouty trait.

## CLINICAL FEATURES

A satisfactory definition of hyperuricemia is difficult to achieve. As with virtually all constituents of serum, the majority of uric acid values in normal subjects are grouped within a relatively small range. The dimensions of the normal range must be defined either statistically or empirically, since there exists no precise criterion for establishing an unequivocal upper limit of the normal range.

### NORMAL URIC ACID VALUE IN SERUM OR PLASMA

Studies on the distribution of serum urate values in the general population must be interpreted with reference to the method employed. In

6.0 mg per 100 ml in males as potentially abnormal. Experience with the Praetorius method is less extensive but in the author's laboratory any value above 6.7 mg per 100 ml in males is considered potentially abnormal since in a comparison of the Folin and Praetorius methods on about fifty samples from normal and gouty subjects values by the latter method have averaged about 13 per cent higher.

#### SERUM URIC ACID VALUES IN GOUT

Using presently acceptable colorimetric methods the serum urate concentration in gout is almost invariably above 6 mg per 100 ml and may be as high as 15 mg per 100 ml though seldom higher. In 177 serum analyses in 21 gouty patients published by Jacobson [11] the urate concentrations ranged from 5.2 to 14.8 mg per 100 ml 98 per cent exceeding 6.0 mg per 100 ml and 91 per cent exceeding 7.0. Talbott and Coombs [12] and Talbott [20] found concentrations ranging from 5.7 to 16.2 mg per 100 ml in over 900 determinations on 100 gouty patients. Over 98 per cent of all values were above 6.0 mg per 100 ml. Goldthwait, Butler and Stillman [20] found values ranging from 4.8 to 16.0 mg per 100 ml in 113 patients with gout but in only 3 were the values below 6 mg per 100 ml and these were in patients with atypical gout.

No large series of gouty patients has been studied with the Praetorius method but several patients with typical gout have had values in the range of 0.8 to 7.5 by this method [27-28]. However these values may have been influenced by dietary factors for protein and purine restriction have been found to lower the serum urate level by 1 to 2 mg per 100 ml after a few days [29].

Data on the frequency of hyperuricemia among relatives of gouty subjects (cf. Genetic Considerations near the end of the chapter) suggest that asymptomatic hyperuricemia is perhaps five times as common as clinical gout.

#### ACUTE GOUT

The most common complication of hyperuricemia is acute gouty arthritis. Primary gout may occur at any age but it is predominantly a disease of adult males. In large series of gouty patients only about 2 per cent are females. Typically an attack occurs without warning in the midst of exuberant health in the mid forties or early fifties. About 50 per cent of initial attacks involve the great toe (podagra); occasionally the initial attack may be bilateral [30]. Ninety per cent of gouty patients experience attacks in the great toe some time during the course of their disease. Next in order of frequency, as sites of initial involvement, are the inner ankle, heel, knee, and wrist. Any joint in the body may at times be involved. Hench [31] has emphasized that the most diagnostic site of involvement, the more typical, is the character of the attack.

TABLE 21.1 SERUM URIC ACID CONCENTRATIONS IN MILLIGRAMS PER 100 ML IN CONTROL SUBJECTS

| Method                                      | Males |          |      |      | Females |          |      |      | Reference |
|---|-------|----------|------|------|---------|----------|------|------|-----------|
|   | No    | Range    | Mean | s.d. | No      | Range    | Mean | s.d. |           |
| Colorimetric (Folin)                        | 63    | 2.0-6.7  | 4.4  | 0.03 | 37      | 1.0-5.9  | 4.0  | 0.11 | [10]      |
| Colorimetric (Benedict)                     | 296   |          | 3.95 | 1.15 | 204     |          | 3.63 | 1.06 | [11]      |
| Colorimetric uricase (Buchanan)             | 49    |          | 5.4  | 1.2  | 53      |          | 4.1  | 0.9  | [12]      |
| Differential spectrophotometric (Practorus) | 143   | 2.0-7.5† | 5.01 | 1.2  | 157     | 2.0-5.7† | 3.84 | 0.93 | [14]      |
| Differential spectrophotometric (Practorus) | 150   | 2.5-9.0  | 5.1  | 0.1  | 150     | 2.2-6.0  | 4.0  | 0.1  | [15]      |

91 M

† Range =  $M \pm 2s.d.$  which included 97 per cent of observations in each sex



Fig 21.1 Chronic gouty arthritis of hands. Note the extensive destruction of bone by urate deposits and the large soft tissue tophi.



Fig 21.2 Chronic gouty arthritis in feet of same patient shown in Fig 21.1.

It is difficult to improve on Sydenham's [32] description of the acute attack.

The victim goes to bed and sleeps in good health. About two o'clock in the morning he is awakened by a severe pain in the great toe—more rarely in the heel, ankle or instep. This pain is like that of a dislocation and yet the parts feel as if cold water were poured over them. Then follow chills and shivers and a little fever. The pain, which was at first moderate, becomes more intense. With its intensity the chills and shivers increase. After a time this comes to its height, accommodating itself to the bones and ligaments of the tarsus and metatarsus. Now it is a violent stretching and tearing of the ligaments—now it is a gnawing pain and now a pressure and tightening. So exquisite and lively meanwhile is the feeling of the part affected that it cannot bear the weight of the bedclothes nor the jar of a person walking in the room. The night is passed in torture, sleeplessness, turning of the part affected and perpetual change of posture, the tossing about of the body being as incessant as the pain of the tortured joint and being worse as the fit comes on. Hence the vain effort by change of posture both in the body and the limb affected to obtain an abatement of the pain.

The attack usually abates spontaneously in 1 to 2 weeks but may last several weeks. Recovery following the initial episode is generally complete.

#### INTERVAL GOUT

The asymptomatic phase may last from a few weeks to many years. Generally in 6 months to 2 years the patient will suffer another episode in the same or another joint and the events of the initial attack will be repeated unless modified by therapy. In the natural history of the disease acute attacks recur with increasing frequency. Eventually gouty arthritis with chronic involvement persists and joint deformity and destruction may occur; these must not be considered simply a later stage of acute gouty arthritis since the two phases have different pathogeneses.

#### CHRONIC GOUTY ARTHRITIS

This is not merely a prolongation of an acute attack. It may begin insidiously and progress with or without the superimposition of further attacks in the involved or other joints. The duration of time from the initial attack to the beginning of chronic symptoms is highly variable, ranging from 3 to 42 years with an average of 11.6 years [33]. In one unique case widespread ankylosis developed following the initial gouty attack [34].

*Characteristic of the chronic stages of gouty arthritis is the accumulation of urate in and around the affected joints. In severe cases the accumulations may be enormous and the deformities of hands or feet grotesque (Figs. 21.1, 21.2).*

The depositions of solid urate are not limited to joint regions nor are they dependent upon prior occurrence of articular gout. Tophi form



Fig 21.1 Chronic gouty arthritis of hand. Note the extensive destruction of bone by urate deposits and the large soft tissue tophi.



Fig 21.2 Chronic gouty arthritis of feet of a patient shown in Fig 21.1.

with some frequency in cartilaginous tissue of the body particularly the helix and antihelix of the ear. Large accumulations may develop in the olecranon or patellar bursae. Rarely, patients may show tophi at the time of the initial attack [35], before uricosuric agents came into general use about 50 per cent developed tophi at some time during the course of their disease [30]. In three large series of gouty subjects recently reported [36-38] the incidence of tophi ranged from 13 to 25 per cent. Tophi were found chiefly in patients with gout of 6 to 10 years' duration or more.

### RENAL IMPAIRMENT

With the passage of time the gouty patient tends to develop renal impairment as a complication of the disease. The advent of renal insufficiency is a particularly serious matter for the gouty subject, for in its presence accumulation of urate is apt to progress at an accelerated pace. After the age of 50 the incidence of tophi is significantly greater when renal damage is present than when it is absent [39].

It has long been appreciated that renal disease is an important cause of death in gout. From 30 to 50 per cent of gouty patients are said to die of renal disease. The most common indications of early renal damage are loss of ability to concentrate solids, mild proteinuria, and a reduction in excretion of phenolsulfonphthalein. Marked proteinuria and retention of uric acid may occur later and are indicative of serious renal involvement and a poor prognosis [39].

### PATHOLOGY

The specific pathologic changes in gout depend upon the deposition of urate in tissues and the associated inflammatory reactions and degenerative changes. The one pathognomonic lesion of gout is the tophus, a urate deposit surrounded by tissue exhibiting inflammatory and foreign body reaction. Because urate crystals are water soluble, nonaqueous fixatives are necessary to preserve the urate deposits in histologic sections. Urate crystals if preserved in bulk are brilliantly anisotropic when viewed with polarized light under the microscope. A useful and simple staining technique is that of de Galantha, in which tissue is fixed in absolute alcohol and subsequently stained with silver [40]. The urate crystals are stained brown black by this method. However, provided the initial fixation is done with absolute alcohol, staining with hematoxylin and eosin is also usually successful, even though the bulk of the crystals may have been dissolved out.

Two general categories of mechanisms have been proposed regarding deposition of urate in gout. Borrowing some terms from concepts of pathologic calcification, Sokoloff [41] has grouped these as follows: (1) *metastatic*, implying that urate is deposited in tissues because excessive quantities are presented to them by circulating blood; (2) *dystrophic*

implying that urate is deposited in tissues because the latter have undergone some primary pathologic alteration rendering them susceptible of urate deposition. These two categories are not mutually exclusive.

### THE TOPHUS

The characteristic tophaceous nodule consists of the multicentric deposit of urate crystals and intercrystalline matrix together with the inflammatory reaction and foreign body granuloma it has evoked (Fig

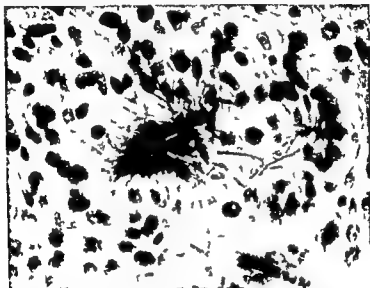


Fig 21-3 Acute gouty synovitis. The tissue has been fixed in absolute alcohol and the section stained by the method of de Clichy. In situ and aggregated needle-like crystals have deposited in incipient tophus formation. This section has been included in The Pathology Teaching Collection of Lantern Slides (By permission of the British and Hebrew Foundations and of Leon Sokoloff).

21-3) [41]. The crystals generally believed to be monosodium acid urate monohydrate [42] are acicular and arranged radially in small clusters. Circular and amorphous deposits have also been described in articular accumulation. Calcific material deposited in the intercrystalline matrix may render the tophus radiopaque. This process though uncommon may at times reach the proportions of heterotopic ossification [43]. A variety of component—protein, lipid and polysaccharide in nature has been described in the gouty tophus. Some workers view the urate deposits as occurring secondary to tissue changes involving such materials. The term *tophus* is occasionally reserved for urate deposits in tissue other than joint although this distinction has no pathologic basis. Tophi are commonly seen in the helix or antihelix of the ear, the olecranon and patellar bursa and tendon. Less frequently they occur in the skin of palm



or soles the tarsal plates of the eyelids nasal cartilages finger tips and in the cornea and sclerotic coats of the eye Rarely they have been observed in the corpus cavernosum and prepuce of the penis, the aorta myocardium aortic or mitral valves the tongue, epiglottis, vocal cords and arytenoid cartilages [30]

It has been pointed out that urates tend to deposit in relatively avascular tissue [30] Tissue alterations of unknown type including evolution of substances in the intercrystalline matrix may play a role in precipitating or maintaining the tophus Such alterations possibly the result of previous injury, might be expected to occur more readily in tissues with a limited blood supply or in tissues such as cartilage which depend on perfusion by body fluids for nutritional requirements Cartilage has an affinity for absorbing urates in vitro [44] and tarsal bones of swine will cause precipitation of needles such as seen in gout when suspended in saturated solutions of urate [40] Sokoloff [41] has pointed out that several sites of urate deposition in gout—articular and other cartilages synovial tissues interstitial tissue of the renal pyramid and sclerae and heart valves—have in common ground substance containing acid mucopolysaccharide If a local pH change is of importance in initiating precipitation of urate from a saturated solution which at pH 7.4 would be comprised of 98.9 per cent monosodium urate and 1.1 per cent uric acid then one might expect that the crystals would actually be uric acid whose solubility is remarkably less than that of its monosodium salt Indeed on crystallographic evidence de Graciansky concluded that the gouty crystalline deposits were probably uric acid rather than the salt [49] This important problem warrants reinvestigation

#### ACUTE GOUTY ARTHRITIS

The mechanism precipitating the acute gouty attack is unknown as is the role of uric acid in this phenomenon A few leads have emerged on the metabolic front in recent years and these will be discussed below Suffice it to say here that many workers [18, 47] entertain serious doubts that uric acid itself is immediately responsible for the local and systemic disturbances in acute gouty arthritis

A question of paramount importance therefore concerns whether urate is deposited in synovial tissues at the time of or immediately prior to the first attack Recently Zeveloff and coworkers [48] found urate crystals in four of six instances in which tissue was obtained by punch biopsy Sokoloff [41] also has reported the occurrence of urate crystals in the synovium during the acute attack There was associated acute synovitis and in Sokoloff's case a submiliary granuloma but no fibrosis These observations were interpreted as evidence that urates are deposited in the articular tissues *during* acute gouty episodes However these patients had had prior attacks and the possibility of earlier deposition

is difficult to exclude. It has also been known for many years that urate deposits may develop in joints in idiosyncrasy and may not give rise to acute symptoms [49-50].

Villa Robecchi and Ballabio [51] obtained a biopsy specimen of synovial membrane from a patient suffering from a first attack of gout. There was acute exudative inflammation without urate deposits. Such negative evidence is of course inconclusive since it offers no information regarding possible crystals elsewhere in the synovium. It is at present uncertain whether the nonspecific acute inflammatory reaction of acute gout is in response to or accompanied by the deposition of urate crystals.

### CHRONIC GOUTY ARTHRITIS

Urate crystals deposit in the synovial membrane, articular cartilage, subchondral bone, capsular and periarticular tissues, tendons, and at times the overlying skin. The crystals are deposited radially in the superficial portions of the articular cartilage. This is generally interpreted as evidence that urates enter the cartilage from the synovial fluid [52].

Synovial involvement becomes more pronounced as the disease advances. A variable degree of nonspecific acute and chronic inflammatory reaction accompanies the tophaceous reaction in the synovial membrane.

The affected portions of the articular cartilage undergo nonspecific degenerative changes. Sokoloff [41] has distinguished three patterns of joint involvement that may occur as a consequence of crystal deposits: (1) disintegration of the articular cartilage may lead to osteoarthritis; (2) urate deposits may cause massive obliteration of joint structures; (3) chronic tophaceous synovitis with the associated inflammatory reaction may lead to small areas of pannus formation rarely rheumatoid-like ankylosis may occur.

No joint is exempt, although joints of the lower extremity are most commonly involved. In vertebral bodies urate deposits are found in marrow spaces adjacent to intervertebral disks as well as in disk tissue itself. The punched-out lesions of bone commonly seen in roentgenograms (Fig. 21-1) of gouty patients represent marrow tophus deposits which in most instances communicate with the urate crust on the articular surface through erosions and defects in the articular cartilage (Fig. 21-4) [41]. The urate deposits of articular cartilage, though at times extensive, lack the inflammatory and foreign body reaction characteristic of the tophus.

Crystals have been recovered from the putum of gouty subjects, but they apparently do not occur in the central nervous system.

### THE KIDNEY IN GOUT

The kidneys of most patients with gout contain deposits of urate in the medulla [53]. The term *gout nephrosis* has been applied to the micro-

tophaceous urate deposits in the renal pyramids [41] Minkowski [24] believed that they were first deposited in the epithelium of the convoluted tubules and were subsequently flushed into the collecting tubules where an inflammatory reaction destroyed the tubular structure. Urate crystals occur frequently in the interstices of the pyramid. Here they evoke an inflammatory reaction and may lead to foci of necrosis [25].



Fig 21-4 Chronic gouty arthritis. Urate crystals in the synovial membrane, articular cartilages, and subchondral bone appear black. Advanced osteoarthritic changes with marginal osteophyte formation are seen. (From L. Sokoloff and I. O. Gleason, *Am J Clin Path* 21: 406, 1954, with permission of authors and publisher.)

In a small proportion of cases the pyramidal deposits of urate are so great as to cause serious mechanical obstruction of the nephrons. This results in atrophy and fibrosis of the tubule with variable secondary chronic inflammatory changes. The end result may be a scarred kidney variously interpreted as obstructive nephropathy or chronic pyelonephritis. Modern and Meister [56] have drawn attention to the combination of progressively increasing azotemia, decreased phenolsulfonphthalein excretion, and a fixed specific gravity of the urine in gouty patients in whom hypertension, albuminuria, and cylinduria are absent. They believe these findings characteristic of the tophaceous kidney of gout.

Evidence for gout nephrosis was cited by Sokoloff [41] in 64 of 80 cases of gout coming to autopsy, but in only 5 cases were the changes considered severe. Arteriolar sclerosis and nephrosclerosis were also commonly seen.

Since renal disease is common in those with recognized gout, one might

anticipate that instances of renal disease should also occur in persons possessing the gouty trait but not manifesting typical clinical gout. Two distinct types of primary renal involvement are worthy of comment.

The first of these is nephrolithiasis. The stones are composed of uric acid, not biurate [56a]. Calculi are said to occur in 12 to 15 per cent of clinically gouty patients [39-57] and are particularly common during uricosuric therapy. However, it is also known that calculi occur with some frequency among hyperuricemic subjects who do not have clinical gout. For example, Boyce (quoted by Talbott [39]) reports an incidence of hyperuricemia of 22 per cent, but of gout of only 10 per cent, among patients passing pure uric acid stones.

The second type of involvement, first described by Ebstein [55], is that of gout nephrosis occurring without clinical evidence of gout. Ueber [58] also described two cases of typical gouty kidneys with no clinical history or other pathologic findings of gout. Brown and Mallory [59] have described one patient who probably falls into this group. In none of the cases were uric acid analyses of blood or serum performed during life.

It is likely that renal disease secondary to clinically occult gout is more common than presently recognized. It should be suspected whenever hyperuricemia seems out of proportion to the degree of renal impairment in patients in whom an adequate cause for the renal disease is not apparent. The suspicion would be enhanced by finding asymptomatic hyperuricemia or clinical gout among family members. A positive diagnosis during life is difficult to achieve, however, since translumbar renal biopsy does not yield the medullary tissue necessary for adequate pathologic study.

#### VASCULAR SCLEROSIS IN GOUT

Arteriosclerosis, especially in the kidney and hypertension, are said to have an increased incidence in gout, particularly in patients who have had their disease for 15 to 20 years [60]. Renal functional impairment is probably attributable to nephrosclerosis in most cases rather than to gout nephrosis [60]. Renal involvement tends to be only slowly progressive [39] and life expectancy and mortality are reportedly normal in gout [61].

### BIOCHEMISTRY OF PURINE COMPOUNDS

#### BIOSYNTHESIS OF THE PURINE RING

Early work concerned with the biosynthesis of purine compounds in animals and man has been reviewed elsewhere [62-65].

A clear distinction should be made between this concept and the point of view that renal insufficiency *per se* may be a cause of gout, as well as of hyperuricemia. Evidence for the latter view is meager and inconclusive. Some workers have suggested that latent gout may be activated by renal insufficiency. These topics are discussed more fully elsewhere [35a].

For many years there was considerable uncertainty regarding the synthesis of the purine ring. Because of structural similarities urea [66], arginine and histidine [67] were proposed as precursors of purines. When experiments were performed with these substances in labeled form [68, 69] and no labeling of tissue purines resulted, these theories were discarded. In 1943 Barnes and Schoenheimer [68] fed ammonium citrate containing  $N^{15}$  to pigeons and demonstrated that the purines of internal organs and the urea acid of the excreta contained appreciable  $N^{15}$  in the ring structure and substituent amino groups. Analogous experiments in rats yielded qualitatively similar data. These pioneer experiments demonstrated that the purine ring was synthesized within the body from small molecules.

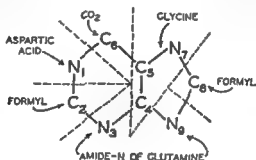


Fig. 21-5 Origins of the atoms of the purine ring (From *Metabolism* 6: 944, 1957 with permission of the publisher.)

Since these early studies rapid strides have been made. The use of various labeled substrates in bacterial, avian, and mammalian systems has clearly defined the origin of the individual atoms of the purine ring. More recently, work with soluble enzyme systems has delineated the biosynthetic sequence in a stepwise manner so that today the metabolic origin of the major purine compounds is well known.

In Fig. 21-5 are shown the precursors of the various carbon and nitrogen atoms in the purine ring. The basic building block is the simplest of amino acids, glycine, which contributes carbon atoms 4 and 5, and nitrogen atom 7 [70-72]. Carbon atoms 2 and 8 come from formate [72-73], carbon atom 6 comes from CO<sub>2</sub> [71-74], nitrogen atoms 3 and 9 come from the amide-N of glutamine [75-77], and nitrogen atom 1 comes from aspartic acid [76]. Compounds which are converted to glycine in the body will also contribute to the 4, 5, and 7 positions of uric acid. Similarly, compounds which donate one-carbon fragments to the "formyl" pool, will contribute to carbons 2 and 8 of purines.

The biosynthesis of purine compounds may logically be considered to begin with the formation of an active ribose derivative,  $\alpha$ -5-phosphoribosyl-1-pyrophosphate, abbreviated PP-ribose-P (Fig. 21-6). This

compound is formed by a transfer of the terminal pyrophosphate group of ATP to the first carbon of ribose 5 phosphate [78-79]



Ribose 5-phosphate has three possible pathways of origin: it may arise as an intermediate in the 6-phosphogluconic acid oxidation pathway of glucose, as a product of nonoxidative cleavage of fructose 6-phosphate, or as a late product of the metabolism of glucose 1-phosphate via the uronic acid cycle. The 6-phosphogluconic acid pathway is probably the major source of ribose esters. (See Chap. 4.)



Fig. 21.6 Biosynthesis of phosphoribosylamine. The encircled numbers in this and subsequent figures refer to numbered reactions in the text.

PP ribose-P is involved in purine synthesis in two types of reactions. One is formation of the first specific intermediate of purine synthesis *de novo*, 5- $\beta$ -phosphoribosylamine [80-82], and the other is direct synthesis of ribonucleotides from the free purine base by a pyrophosphorylase mechanism.

In the former reaction the pyrophosphate group of PP ribose-P is displaced by the amide group of glutamine, and there is an inversion of the substituent to yield the  $\beta$  linkage characteristic of the glycosidic bond of all known ribonucleotides [82].



The exact mechanism of this reaction is not yet understood, but both PP ribose-P and glutamine are bound independently by the enzyme prior to the reaction [83]. The reaction generating phosphoribosylamine is the first irreversible reaction dealing specifically with synthesis of purines *de novo*, and it thus becomes a potentially important reaction from the standpoint of control of purine synthesis.

The stepwise synthesis of the purine ring is shown in Fig. 21.7. Phosphoribosylamine reacts with glycine to yield glycinamide ribonucleotide. ATP is involved in this reaction as an energy source [84-86].



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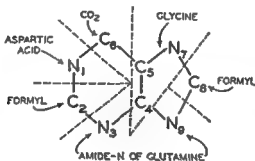


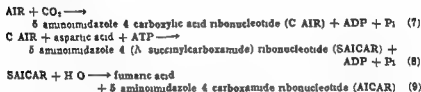
Fig. 21-5 Origins of the atoms of the purine ring (From *Metabolism* 6:244, 1957 with permission of the publisher)

Since these early studies rapid strides have been made. The use of various labeled substrates in bacterial, avian, and mammalian systems has clearly defined the origin of the individual atoms of the purine ring. More recently, work with soluble enzyme systems has delineated the biosynthetic sequence in a stepwise manner so that today the metabolic origin of the major purine compounds is well known.

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5-Aminimidazole ribonucleotide (AIR) now receives a carboxyl group at C<sub>4</sub> by a CO<sub>2</sub>-fixation reaction [92] which probably involves biotin as a coenzyme [93]. The carboxyl serves as a point for condensation of this intermediate with aspartic acid through an amide linkage involving another ATP as the source of energy [94]. Hydrolysis of the intermediate now yields 5-aminimidazole-4-carboxamide ribonucleotide, a compound lacking only the number 2 carbon atom of a complete purine ribonucleotide.



AIC-ribonucleotide receives a second formyl group from a tetrahydrofolic acid derivative [95-96] and ring closure completes the biosynthesis of the purine structure by forming hypoxanthine ribonucleotide or inosine 5-monophosphate (IMP) as it is usually termed [96].



One intermediate in the synthesis of IMP deserves special comment viz 5-aminimidazole-4-carboxamide ribonucleotide. Its free base 4(5)-aminimidazole 5(4)-carboxamide (AIC) was first isolated in 1945 by Stetten and Fox [97] from cultures of *Escherichia coli* grown in the presence of sulfonamide. The elucidation of its structure by Shive and coworkers [98] stimulated considerable interest since it was immediately recognized as a potential purine precursor. This postulate was confirmed when Miller, Curin, and Wilson [99] demonstrated incorporation of AIC-C<sup>14</sup> into purines and purine catabolites in the rat. Other studies further substantiated the utilization of AIC for purine synthesis [100]. It eventually became clear, however, that it was not free AIC but rather its ribonucleotide which was the direct precursor of IMP [101-102]. Labeled AIC has proved to be an extremely useful compound in studies of purine synthesis in gouty subjects as discussed below.

## BIOSYNTHESIS OF OTHER NUCLEOTIDES

IMP may be considered the parent purine compound. It has several possible fates (Fig. 21-8). First to be considered here is its role as an intermediate in the formation of adenosine 5-monophosphate (AMP) and





AMP and GMP may be converted to di- and triphosphates which are essential coenzymes of many reactions and building blocks for nucleic acids

### RECONSTITUTIVE REACTIONS

Mechanisms exist for resynthesis of ribonucleotides from free bases or ribonucleosides both of which must have arisen from prior cleavage of nucleotides synthesized *de novo* by pathways just discussed

Free purine bases may be converted to ribonucleotide form by reacting directly with PP ribose-P according to the following scheme



Two different purine ribonucleotide pyrophosphorylases have been identified one acting on AIC and adenine [112] another on hypoxanthine, guanine and 6 mercaptopurine [113-114]. These enzymes are widely distributed and have been studied most extensively in yeast [107], beef liver [112-113] and human erythrocytes [114].

A second mechanism involves conversion of base to ribonucleoside by reversal of the nucleoside phosphorylase reaction [115-117] and subsequent conversion of ribonucleoside to ribonucleotide by action of the appropriate kinase [118, 119] according to the following schemes



Nucleoside phosphorylases are widely distributed in mammalian tissue and are particularly active in liver [117] and erythrocytes [120]. At least in the case of inosine the equilibrium point is far toward the ribonucleoside [116]. Nucleoside kinase reactions have been studied less extensively in mammalian systems but do appear to exist. For example Goldthwait [121] found adenosine to be converted to acid soluble nucleotides by dog heart muscle even more actively than was adenine. Similarly Lowy et al [122] have shown that AIC ribonucleoside is readily utilized by rabbit erythrocytes for synthesis of ATP and GTP.

Reutilization of purine bases and nucleosides released by catabolism of nucleic acids and coenzyme nucleotides has been viewed by Kornberg [123] as a salvage process. By either of these reconstitutive pathways only one high-energy bond in the form of PP ribose-P or ATP is expended in the synthesis of a nucleotide whereas in the synthesis of IMP *de novo* from glycine and PP ribose-P a minimum of four ATP molecules is required.

In addition to the salvage function performed by these reactions it is probable that they are also important primary biosynthetic mechanisms in some tissues [124] which are dependent upon a supply of preformed

guanosine 5' monophosphate (GMP), the purine nucleotide components of nucleic acids. IMP is aminated in the 6 position to form AMP [103]. This conversion occurs in two steps involving an initial condensation of IMP with aspartic acid to form adenylosuccinic acid (AMP-S) [104, 105]. The energy necessary for this reaction is derived from the cleavage of

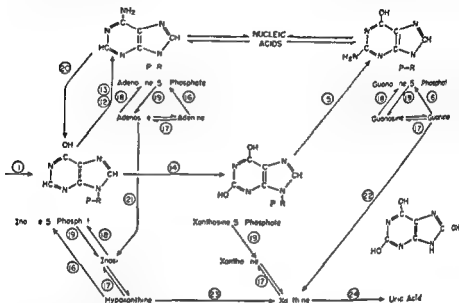
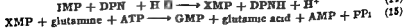
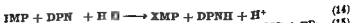


Fig. 21.8 Biosynthesis of purine ribonucleotides, deoxyribonucleotides, and bases

guanosine triphosphate [106] but the reaction is complex and its mechanism poorly understood [106-107]. Hydrolysis of AMP-S yields AMP and fumaric acid. This latter reaction is freely reversible [106].



The conversion of IMP to GMP also occurs in two steps. The first is irreversible oxidation of IMP to xanthosine 5' monophosphate (XMP) with diphosphopyridine nucleotide (DPN) as hydrogen acceptor [108, 109]. The second is the amination of XMP at position 2 and the specific amino donor for the reaction is the amide group of glutamine [110-111]. This step requires ATP as the source of energy. These reactions proceed according to the overall schemes shown below.



fraction of pigeon liver reduced synthesis of IMI from glycine by 90 per cent [126]

### CONTROL OF SALVAGE PATHWAYS

Way and Parks [131] have shown that the conversion of certain purine analogues to their respective ribonucleotides by the pyrophosphorylase reaction (Reaction 16) is also strongly inhibited by ADP or ATP. The results suggest the existence of another negative feedback control suppressing the formation of new nucleotides from purine bases when adequate concentrations of nucleotides are already present.

### CONTROL OF RIBONUCLEOTIDE INTERCONVERSION

The cofactor in the conversion of IMP to AMP is GTP whereas the cofactor in the conversion of AMP to GMP is ATP. Stetten [132] has suggested that the reciprocal roles of the triphosphates in the formation of the monophosphates may help preserve a balance between AMP and GMP synthesis by a positive feedback control.



In a bacterial system studied by Magasanik the conversion of IMP to AMP is inhibited by CMP or other guanine compounds [133]. A potential feedback regulation of GMP synthesis from IMP is thus disclosed. 6-Mercaptopurine ribonucleotide has also been shown to inhibit conversion of IMP to AMP as well as conversion of IMP to AMP [134]. This latter conversion is also inhibited by 2,6-diaminopurine or derivatives thereof [135]. These results suggest the possible existence of a control of AMP synthesis from IMP by natural ribonucleotides, but this awaits demonstration. In a bacterial system the synthesis of AMI from IMP is regulated in a novel way. In the sequence  $\text{IMP} \longrightarrow \text{AMP} \longrightarrow \text{GMP} \longrightarrow \text{IMI}$  the terminal reaction is strongly inhibited by ATP [133]. This cycle is not known to occur in mammalian systems.

The feedback controls presently demonstrated as potentially operating on nucleotide interconversions are shown in Fig. 21.9.

In pigeons and other uricotelic organisms the unrestrained synthesis of IMI is the major process for disposal of waste nitrogen and results in generation of large quantities of hypoxanthine and ultimately of uric acid rather than overproduction of nucleic acids. Evidence to be presented below suggests that an appreciable amount of uric acid is also synthesized in normal man by such a *de novo* pathway and that abnormal amounts may be made in certain gouty subjects by this or other routes which by-pass nucleic acids. The mechanism whereby IMP is

purine precursors for their nucleic acid synthesis Lajtha and Vane [125] have demonstrated that bone marrow, for example is dependent upon a preformed purine precursor originating in liver for its source of nucleic acid purine bases The exact form of the precursor is not identified

## NORMAL CONTROL MECHANISMS

### REGULATION OF PURINE SYNTHESIS DE NOVO

The first irreversible reaction concerned specifically with purine synthesis is that in which  $\beta$  phosphoribosylamine is generated from  $\alpha$  phosphoribosylpyrophosphate and glutamine [80-82] by the enzyme named phosphoribosylpyrophosphate amidotransferase [82] Phosphoribosylamine has no known function other than its role in purine synthesis *de novo* It is however, an unstable compound and is readily broken down if not promptly utilized Because of these factors the rate-limiting step in purine biosynthesis may be the reaction generating phosphoribosylamine [83-126]

The rate of generation of phosphoribosylamine is in turn dependent upon the activity of the amidotransferase and the abundance of glutamine and PP ribose-P Activity of the amidotransferase is influenced by certain purine ribonucleotides which are competitive inhibitors of binding of PP ribose-P but not of glutamine by the enzyme ADP and ATP are the most potent of the inhibitory ribonucleotides and the dissociation constants of their enzyme-inhibitor complexes are some six times the  $K_m$  of PP ribose-P [83] A potential feed back control of purine biosynthesis *de novo* by purine ribonucleotides is thus disclosed since ADP and ATP are present in cells in appreciable concentration [127-128] and PP ribose-P is probably present in relatively low concentration No natural factors which compete with glutamine for binding by the enzyme are known and it is unlikely that glutamine is involved in a regulatory mechanism The effects of high protein diets in augmenting purine synthesis [129] may operate through glutamine however since its  $K_m$  in this reaction is quite high  $1.1 \times 10^{-3}M$  (pigeon liver enzyme)

Factors tending to lower concentrations of substrates may also reduce phosphoribosylamine synthesis and thus reduce purine synthesis *de novo* Some factors have been studied with respect to PP ribose-P Since this ester is also utilized in reactions which generate ribonucleotides from purine and pyrimidine bases directly the availability of PP ribose-P for purine synthesis *de novo* will be influenced by the balance achieved between rates of synthesis of PP ribose-P and of utilization in various ribonucleotide pyrophosphorylase reactions The inhibition of purine synthesis *de novo* by preformed purine bases [126-130] may represent chiefly diversion of PP ribose-P from the *de novo* sequence For example inclusion of adenine ( $2 \times 10^{-4}M$ ) in an incubation mixture of the soluble

quence Although these substances act as glutamine antagonists in each of the three reactions of purine synthesis in which glutamine is a substrate [81 111 136] the step involving conversion of  $\alpha$ -N formyl glycinamideribonucleotide to the corresponding amidine compound is by far the most sensitive to these agents [111 136]



Azaserine [137] and DON [138] also inhibit glycine incorporation into uric acid in man presumably at this reaction site

## NUCLEIC ACID CATABOLISM

Enzymatic hydrolysis of the polynucleotide chains of nucleic acids occurs through the action of various nucleases [139] The major products released by ribonuclease a and b and by deoxyribonuclease I and II are oligonucleotides The oligonucleotides are further cleaved by phosphodiesterases to yield 5- and 3' mononucleotides

The mononucleotides are split by group specific nucleoside 5' phosphatases [140] as well as by a variety of nonspecific phosphatases [141] to yield the corresponding purine or pyrimidine nucleoside and orthophosphate The purine nucleoside is then split by purine nucleoside phosphorylase [115] to yield the free purine base and ribose or deoxyribose 1 phosphate [115 116]



Analogous reactions exist for pyrimidine nucleosides but since there is presently no evidence that pyrimidine compounds play a role in gout they will not be considered in this chapter

In addition to these general reactions AMP and adenosine are acted upon by specific deaminating enzymes AMP is converted to IMP by adenylic deaminase (Reaction 20) [142] and adenosine to inosine by adenosine deaminase (Reaction 21) [143] (Fig 21-8)

## SUBSTITUTED PURINES

Recently certain other bases have been shown to exist as minor constituents of nucleic acids The mononucleotides derived from DNA contain small quantities of 5-methylcytosine 5-hydroxymethylcytosine and 6-methylaminopurine in addition to adenine guanine thymine and cytosine [144] Those derived from RNA contain small quantities of thy

shunted into cleavage fates away from additional synthesis of AMP and GMP may involve feed back regulation of the further synthetic reactions as suggested above

The possibility also exists that conversions of IMP to AMP and XMP are already proceeding at near maximal rates because of enzyme saturations at low substrate concentrations of IMP. Concentrations of IMP in excess of those capable of influencing further ribonucleotide synthesis might then result in increased production of hypoxanthine especially if the  $K_m$  value of the nucleotide 5 phosphatase which initiates the

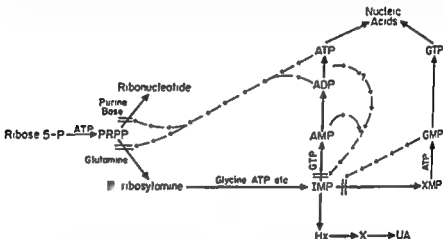


Fig. 21.9 Potential feed back control of purine biosynthesis

cleavage sequence were rather high. No  $K_m$  data are available in avian or mammalian systems from which to evaluate this point.

#### OTHER FACTORS

It seems unlikely that any of the substrates which serve as specific donors of atoms of the purine ring would be limiting with respect to purine synthesis since they are present in abundance within the body and the fraction of their total turnover ultimately employed in purine synthesis must be small indeed. Similarly it is unlikely that specific cofactors would be limiting. Finally the activities of the various enzymes of the biosynthetic sequence eventually converting phosphoribosylamine and glycine to IMP do not appear to be limiting for the intermediates of this sequence normally do not accumulate and the corresponding ribonucleosides or aglycones do not appear in urine.

No evidence was secured for any normal regulatory mechanism operating between glycinamideribonucleotide and IMP in pigeon liver extracts [126]. The antibiotics azaserine and 6-diazo 5-oxo-L-norleucine (DON) do, however, inhibit purine biosynthesis at a site in this portion of the

oxidized by the same enzyme to yield uric acid (Reaction 24) [152] (Chap 22) It will be noted from Fig 21 8 that whereas adenine hypoxanthine and guanine appear to be derived exclusively by cleavage of the corresponding nucleoside xanthine has at least three direct precursors namely its nucleoside (xanthosine or deoxyxanthosine) free hypoxanthine and guanine

Until recently the only established reaction generating uric acid was the oxidation of xanthine A second potential pathway, involving phosphorytic cleavage of uric acid riboside to uric acid and ribose 1 phosphate has now been demonstrated [153] The phosphorylase capable of catalyzing this cleavage is different from the general purine nucleoside phosphorylase and is specific for uric acid riboside among purine compounds but it will also cleave thymidine This finding raises a question as to the identity of its natural substrate Also unsettled is whether this enzyme is different from the thymidine phosphorylase discovered in kidney by Friedkin and Roberts [154] The occurrence of uric acid riboside is well established only in beef erythrocytes [155] and liver [156] Several workers have failed to find the compound in human erythrocytes [157, 158] though it was originally claimed to be there [159] The mode of biosynthesis of this compound is unknown and its significance is at present uncertain Nevertheless it constitutes a potential precursor of uric acid of considerable interest

## PURINES IN BIOLOGIC FLUIDS

### PLASMA

The predominant purine of plasma is uric acid The  $pK_A$  and  $pK_A$  of uric acid are 5.75 and 10.2 respectively [160] Therefore at pH 7.4 about 98 per cent of uric acid will exist as the monoalkali salt [161] assuming no complex formation and no physical binding by large molecules There is little evidence in man for aggregation of uric acid either with itself or with large molecules Dialysis equilibration studies have shown that less than 4 per cent of uric acid is bound to nondiffusible elements at plasma levels ranging from 2 to 7 mg per 100 ml [163] Ultrafiltration studies have shown that virtually all plasma urate of normal and gouty subjects is readily ultrafiltrable [163] In electrophoresis of plasma neither the natural uric acid originally present in plasma nor that added to it migrates with protein in both cases uric acid moves anodally ahead of albumin [164] These results have thus failed to confirm the presence of a uric acid-protein complex in blood [165, 166]

Mono sodium urate is soluble in distilled water to the extent of approximately 100 mg per 100 ml [167] Its solubility in serum is considerably less probably because of effects of other ions present [168] Solubility of uric acid in serum in vitro has been reported as high as 20 mg per 100 ml



mine 2 methyladenine 6 methylaminopurine 6 dimethylaminopurine  $N^2$  methylguanine (6-hydroxy 2 methylaminopurine) and 1 methylguanine [145, 146] in addition to adenine guanine uracil and cytosine. Thus far none of these new bases has been found in mammalian nucleic acids except for 6 methylaminopurine, which occurs in rabbit and rat liver [146]. However since certain of them are found in human urine in small quantities the possibility exists that they may also occur in nucleic acids of man.

### CATABOLISM OF INGESTED NUCLEOPROTEINS

Nucleic acids of dietary nucleoproteins are liberated in the intestinal canal by the action of proteolytic enzymes. Nucleic acids are degraded in turn to nucleotides by nucleases and phosphodiesterases secreted by the pancreas. The nucleotides are chiefly hydrolyzed to nucleosides by various nucleotidases and phosphatases and the nucleosides may be absorbed intact or they may be cleaved phosphorolytically to yield the free base. The small intestinal mucosa of man is rich in nucleoside phosphorylase and xanthine oxidase [147] so ingested nucleoprotein purines may potentially be converted to uric acid prior to entrance into the blood stream. From experiments in which normal and gouty subjects ingested  $N^{15}$  labeled nucleic acids [148] it appeared that the purine moieties were converted to uric acid largely by direct routes without prior incorporation into body nucleic acids. These results accord with the information presented above and also with the observation that persons placed on purine free diets show a prompt fall in urinary uric acid excretion reaching stable low values in 2 to 4 days. That small quantities of dietary nucleosides and even nucleotides may be utilized directly for synthesis of nucleic acids and other metabolic functions in the body is also suggested by recent work [149].

### FORMATION OF URIC ACID

The free purine bases that result from nucleoside cleavage are adenine, guanine, hypoxanthine and xanthine. Since purine nucleoside phosphorylase acts most readily upon inosine and guanosine [115, 117, 150] the major bases generated are very likely hypoxanthine and guanine. In mammalian tissue free adenine is not deaminated as it is in certain bacterial systems [141] so that adenine does not give rise directly to hypoxanthine. If it is not reconverted to its nucleoside or nucleotide it may be excreted unchanged and normal human subjects excrete adenine in small quantities in urine [151]. In contrast the other purine bases are readily converted to uric acid. Guanine is deaminated by guanase to yield xanthine (Reaction 22). Hypoxanthine is oxidized by xanthine oxidase to yield xanthine (Reaction 23) which in turn is further

Uric acid riboside exists in beef erythrocytes but modern chromatographic methods have failed to confirm its presence in human erythrocytes

Uric acid itself is present in erythrocytes but its concentration in red cell water is only about one half that found in plasma Presumably uric acid enters the cell by diffusion and added urate is distributed in serum and erythrocytes in the same proportion as naturally occurring urate [65]

#### SPINAL FLUID

Uric acid is very low in spinal fluid [162] ranging in normal subjects from 0.25 to 1.0 mg per 100 ml The low concentration has been attributed to selective impermeability of the blood brain barrier to urates Values are said to be slightly higher in children (0.3 to 1.5 mg per 100 ml) and to be increased in all forms of meningitis The low levels of uric acid in spinal fluid probably explain the absence of tophaceous deposits in the central nervous system even in advanced cases of gout

Succinoadenine ribonucleoside has recently been identified in spinal fluid [183]

#### JOINT FLUID

Uric acid exists in joint fluid in concentrations approximately equal to those of plasma both in normal and hyperuricemic subjects In patients with tophaceous gout uric acid crystals may be present in joint fluid [52]

#### GASTROINTESTINAL FLUIDS

Uric acid occurs in variable concentration in saliva gastric and pancreatic secretions intestinal juices and bile Much of the early data on uric acid concentrations in these fluids is reviewed by Bishop and Talbott [6.] Recently Sorensen [184] reinvestigated this topic and found values sufficient to account for entrance of about 200 mg uric acid into the gut per day in persons with normal serum values of uric acid

#### BODY WATER

The theoretic uric acid space or volume of distribution of the miscible pool of uric acid at a concentration equal to that of plasma is about one half that of total body water [185] This suggests that portions of body water are considerably poorer in uric acid than is plasma One such large compartment may be muscle water in which uric acid concentrations are very low [63] Another smaller compartment is spinal fluid

#### URINE

The urine of a normal adult on a low purine diet normally contains 270 to 600 mg uric acid per day Values are somewhat lower in women than in men The mean values ranged from 320 to 483 mg per day in seven published reports summarized by Gutman and Yu [18]

[167] However the author has observed levels ranging from 43 to 68 mg per 100 ml [169] on several occasions in patients with acute leukemia treated with cortisone. The possibilities of polymeric complex formation or of unusual binding by abnormal proteins released into plasma in these instances deserve attention.

The only other purine base known to exist in plasma is hypoxanthine although small amounts of xanthine may also be present. In freshly drawn blood the "oxypurine" concentration ranges from 0.1 to 0.4 mg per 100 ml plasma [170-171]. The hypoxanthine in plasma is believed to originate from nucleotides of erythrocytes. In blood which has stood for 48 hr a hundredfold increase in hypoxanthine and xanthine has been demonstrated [172]. Erythrocytes contain enzymatic equipment to degrade adenyl nucleotides to IMP or adenosine and eventually to hypoxanthine, but since erythrocytes and plasma are devoid of xanthine oxidase activity, hypoxanthine is not further metabolized in blood. It is removed either by renal excretion or by extraction by various tissues in which it is then reutilized for nucleoside or nucleotide synthesis or oxidized to uric acid.

There is no information to suggest that nucleosides or nucleotides are normal constituents of plasma [173-175]. In hypotensive shock resulting from crush injuries to muscle a compound appears in plasma which has many characteristics to suggest that it is inosinic acid [176]. Adenylic acid has not been found under these or other circumstances. It seems probable that purine bases and ribosides may enter plasma under special circumstances. For example excretion of inosine, guanosine [177] and adenosine [178] has been observed in urine in certain subjects suggesting prior transport to the kidney by blood plasma. Similarly, about ten purine bases regularly appear in urine [151, 179-180] certain of which may show alterations of excretion in various disease states such as gout, polycythemia vera, leukemia or pernicious anemia. It seems reasonable to presume that they exist in plasma in minute concentrations even though they have not yet been demonstrated there. Also the purine precursor utilized by bone marrow for purine synthesis postulated by Lajtha and Vane [125] must be conveyed in the plasma.

#### ERYTHROCYTES AND LEUKOCYTES

Under normal circumstances nucleotides of blood exist exclusively in erythrocytes and leukocytes although they have been identified in serum in leukemia and hemolytic anemia [174]. The concentration of nucleotides in whole blood normally ranges from 2 to 6 mg nitrogen per 100 ml [181-182]. The purine compounds of erythrocytes and leukocytes [127-170] have recently been studied intensively and a variety of compounds identified. ATP levels average 433  $\mu$ moles and ADP 54  $\mu$ moles per liter of whole blood.

8 Hydroxy 7 methylguanine does not arise by action of xanthine oxidase on 7 methylguanine [187] so that its origin too is obscure although its structure precludes existence as an N9 ribosyl derivative. Of methylated

TABLE 21.2 URINARY EXCRETION IN MILLIGRAMS PER DAY OF PURINE BASES IN NORMAL SUBJECTS

| Purine base                  | Range    | Mean |
|------------------------------|----------|------|
| Hypoxanthine                 | 5.9-13.2 | 9.7  |
| Xanthine                     | 5.1-8.6  | 6.1  |
| Adenine                      | 1.1-1.7  | 1.4  |
| Guanine                      | 0.2-0.6  | 0.4  |
| 1 Methylhypoxanthine         | 0.7-0.7  | 0.4  |
| 8 Succinoaminopurine         | 0.8-1.5  | 1.2  |
| 1 + 7 Methylguanine          | 5.0-7.8  | 6.5  |
| N <sup>2</sup> Methylguanine | 0.4-0.6  | 0.5  |
| 7 Methyl-8-hydroxyguanine    | 1.1-2.0  | 1.0  |

SOURCE: B. Weimann et al. [181] and [180].

purine bases found in human urine only N<sup>2</sup> methylguanine and 1 methylguanine have been found as trace constituents of nucleic acids in both cases in yeast RNA [145].

### BIOSYNTHESIS OF SUCCINOADENINE

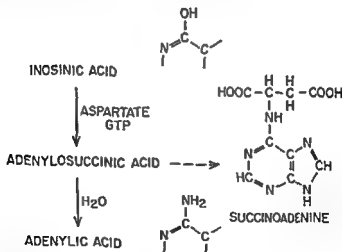


Fig. 21.11. Origin of succinoadenine.

In addition to the compounds shown in Fig. 21.10 at least three unidentified compounds found in human urine appear to be purines on the basis of their absorption spectrums [179]. Also in some individuals on a

The urine contains a variety of purine compounds in addition to uric acid although the total of these bases amounts only to some 30 mg per day [151, 179-180]. The formulas of compounds presently identified and believed to be of endogenous origin, are shown in Fig. 21.10. Their normal excretion values are given in Table 21.2.

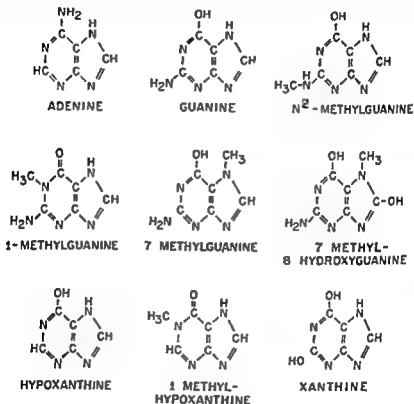


Fig. 21.10 Purine bases of human urine. Succinoadenine (Fig. 21.11) is an additional component.

The metabolic origins of some of these compounds are well understood and of others are completely unknown. The origins of adenine, guanine, hypoxanthine, and xanthine have already been discussed. Succinoadenine is the aglycone of adenylosuccinic acid [104], the intermediate in the conversion of IMP to AMP (Fig. 21.11). The origins of methylated derivatives are unknown. N<sup>2</sup>-Methylguanine may be formed from guanine at the free base level by a reaction analogous to that forming the N-methyl derivative of 2,6-diaminopurine in *Escherichia coli* [186], but this is not established. It appears likely that methylation of guanine to 7-methylguanine occurs at the free base level, for only in free form does guanine contain a replaceable hydrogen at N7, but again this is not known.

addition of unlabeled uric acid may be estimated. In the normal subject this addition may be presumed to represent newly synthesized uric acid.

In 12 normal male subjects studied by this technique [184 188-193] the miscible pool was an average of 1 176 mg uric acid with values ranging from 866 to 1 587 mg. In one normal female the pool was 650 mg [185]. These values confirmed earlier analytical values of Gudzent [194].

From the rate of decline in concentration of  $N^{15}$  or  $C^{14}$  in urinary uric acid it was calculated that from 45 to 85 per cent of uric acid of the miscible pool was normally being replaced each day by newly formed nonisotopic uric acid. These values corresponded to quantities of uric acid ranging from 500 to 1 100 mg per day. In each case the quantity of uric acid entering the pool exceeded the quantity leaving it in urine by 100 to 260 mg uric acid per day [184 188]. The significance of this surplus will be discussed below.

Determinations of the miscible pool of uric acid in patients suffering from gout have yielded values ranging from 1 500 to 31 000 mg [184 188 190 192 195]. The low value [195] is within the range of the highest values found in normal subjects. However in most gouty subjects the miscible pool is decidedly greater than normal the extreme value of 31 000 mg being some twenty five times the average normal value.

The miscible pool of uric acid in gouty subjects may be far greater than can exist in solution in body water in view of the known relative insolubility of uric acid in water at physiologic pH and temperature. The superficial layers of urate masses situated as tophi may contribute to the miscible pool of uric acid. Study of a tophus excised in the course of an isotope experiment in a severely gouty subject demonstrated [190] that peripheral layers of the tophus did indeed contain uric acid enriched with  $N^{15}$ .

The demonstration of large increases in quantities of miscible uric acid present in gouty individuals merely confirms what every clinician already knew and by itself gives no indication of the nature of the disturbance which leads to this increase. However the critical information relating to rate of synthesis of uric acid in gouty individuals has proved elusive for in gouty individual dilution of isotope in circulating urate may occur as a consequence of slow exchange with old urate as well as of addition of newly synthesized urate. It is therefore generally not possible to determine the rate of generation of uric acid by the isotope dilution principle in gouty subjects. An exception to this statement may exist in the few gouty subjects in whom all of the miscible uric acid may be calculated to be in solution. In two such patients Sorenson [184] recently found evidence for increased turnover of uric acid from which he inferred that hyperuricemia was due to increased synthesis of uric acid in these subjects.

The rate of generation of uric acid has been studied in both normal and

methypurine free diet the high excretion values of methylated xanthine derivatives suggest that these bases may at times be partly of endogenous origin

## METABOLIC DEFECTS OF GOUT

### ORIGIN OF HYPERURICEMIA

The accumulation of uric acid within the bodies of patients with gout may be considerable even in the absence of clinically detectable tophi or roentgenographic evidence of such deposits. In advanced cases uric acid may be present in kilogram quantities. Clearly these accumulations of urate must result from a derangement or more than one, in the normal balance between rates of urate formation and rates of urate excretion and destruction. The possible causes of elevated urate concentrations in patients with gout have intrigued students of this disease for half a century and evidence has been advanced in support of each theoretically responsible factor. Even today no clear cut explanation applicable to all cases can be offered, it may be that so-called primary gout is a heterogeneous disorder. In the following sections the available evidence dealing with primary metabolic defects of gout will be considered.

### *Uric Acid Production*

In the past decade the mechanisms and rates of uric acid production have been under intensive study by means of two general types of biochemical techniques (1) the turnover study employing principles of isotope dilution (2) precursor administration with evaluation of the rate and extent of conversion of the administered substance to uric acid.

In the first [188] isotopic uric acid is injected intravenously and permitted to mix intimately with uric acid in the body of the subject. Urinary uric acid is isolated serially for several days and the isotope concentration of each sample is determined. Values are plotted on semi-logarithmic coordinates and the theoretical concentration of isotope in the body at the moment of mixing is obtained by extrapolation of the decay curve to zero time. The miscible pool of uric acid is defined as that quantity of uric acid in the body of the recipient by which the injected uric acid is promptly diluted. The quantity of uric acid present in the miscible pool may be readily calculated from knowledge of the quantity of uric acid injected, of the concentration of isotope in it, and of the concentration of isotope in the uric acid of the body at the moment of mixing.

After mixing has occurred a further progressive decline in concentration of isotope in uric acid occurs because of continuous dilution of the labeled uric acid pool by newly synthesized nonisotopic uric acid molecules. From the rate of this decline in isotope concentration the rate of

[197-199] have conformed with those presented above. However Bishop Rand and Talbott [198] described one gouty patient who incorporated excessive quantities of glycine  $\text{C}^{14}$  into urinary uric acid on two of three occasions despite a normal urinary uric acid excretion.

In studies [27-200] by the author employing glycine  $\text{C}^{14}$  6 of 8 gouty subjects and 1 asymptomatic hyperuricemic patient showed cumulative incorporation of  $\text{C}^{14}$  into urinary uric acid two to fivefold greater

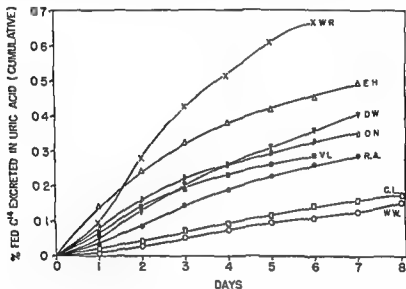


Fig 21.13 Cumulative excretion of  $\text{C}^{14}$  in urinary uric acid following oral administration of glycine  $\text{C}^{14}$  (From *J Clin Invest* 36:1509, 1957 with permission of the publisher)

than found in 3 control subjects (Fig 21.13). Only 2 of the 8 gouty subjects excreted uric acid in quantities clearly in excess of normal. Seegmiller et al [28] and Gutman and associates [199] found normal incorporation values of glycine  $\text{C}^{14}$  or glycine  $\text{C}^{14}$  in gouty or asymptomatic hyperuricemic subjects who did not excrete abnormally large quantities of uric acid in urine. The results of all published studies employing glycine  $\text{C}^{14}$  are summarized in Table 21.3A. Values for incorporation into uric acid in 7 controls ranged from 0.11 to 0.22 per cent in 7 days (average 0.17 per cent). Values were 0.25 per cent or above in 7 days in 6 of 10 gouty patients and in 1 asymptomatic hyperuricemic subject who excreted normal quantities of uric acid in urine under basal conditions. In contrast they were 0.25 per cent or more in 7 days in the 3 gouty subjects and the hyperuricemic subject with renal lithiasis who habitually excreted larger than normal amounts of uric acid in urine under basal



gouty subjects by observing the rate at which isotope appeared in uric acid when a labeled precursor was administered. Studies of this type have now been performed with glycine  $N^{15}$ , glycine 1  $C^{14}$ , glycine 2  $C^{14}$ , formate  $C^{14}$ , 4 aminimidazole 5 carboxamide 4  $C^{13}$  and 4  $C^{14}$  hypoxanthine 8  $C^{14}$  and adenine 8  $C^{14}$ .

The first studies were performed by Stetten and associates [195, 196] employing glycine  $N^{15}$ . When isotopic glycine was fed to normal subjects the  $N^{15}$  concentration in urinary uric acid rose slowly to a maximum after

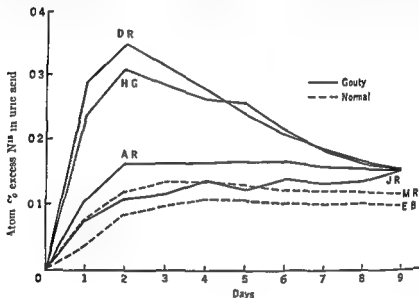


Fig 21.12 Concentration of  $N^{15}$  in urinary uric acid following ingestion of glycine  $N^{15}$  by normal and gouty subjects (From J. D. Benedict et al [196] with permission of authors and publisher)

3 to 4 days and declined gradually thereafter. After 10 days about 0.15 per cent of the administered isotope had been excreted as uric acid. Two gouty subjects who consistently excreted excessive quantities of uric acid in urine and therefore could be presumed to be overproducers showed rapid rises in  $N^{15}$  concentration in uric acid to values about three times normal and fairly rapid declines in  $N^{15}$  concentration during the succeeding days (Fig 21.12). This reflected an exaggeration of the rate of incorporation of dietary glycine nitrogen into uric acid and this finding was borne out by a cumulative incorporation of  $N^{15}$  into uric acid three times greater than normal in these subjects. However, three other gouty subjects whose basal uric acid excretions were within the normal range were also normal as to both rate and magnitude of uric acid labeling.

Since these initial studies by Stetten and associates a number of additional studies has been performed with glycine- $N^{15}$ . Most of the results

compares gouty subjects as a group with nongouty subjects there is significant overincorporation of glycine 1 C<sup>14</sup> into urinary uric acid in those with normal as well as in those with excessive urinary excretion of uric acid (Table 21 3B)

It is probable that even those gouty subjects whose incorporation data are not distinguished from normal are in many instances actually overincorporating glycine into uric acid. Perhaps when appropriate corrections are made for varying degrees of dilution within the uric acid

TABLE 21 3B STATISTICAL ANALYSIS OF DATA OF TABLE 21 3A

| Subjects                       | No | Incorporation of<br>glycine 1 C <sup>14</sup> into<br>urinary uric acid<br>per cent in 6-7 days | t (vs<br>controls) | p (vs<br>controls) |
|--------------------------------|----|---|--------------------|--------------------|
| Controls                       | 8  | 0.17 ± 0.04†  |                    |                    |
| Gouty normal urate excretion   | 11 | 0.29 ± 0.12   | 2.79               | <0.02              |
| Gouty elevated urate excretion | 4  | 0.65 ± 0.36   | 3.87               | <0.01              |

The cumulative incorporation values at 6-7 days have been averaged for three groups: controls (including subject L. H. M. whose uric acid values are normal), gouty subjects with normal urinary urate excretions (including subject R. A.) and gouty subjects with elevated urinary urate excretion (including subject T. J. B.)

† Mean ± 1 s.d.

pool and for uricolysis a more definite statement can be made. For example a gouty subject (e.g. L. O. Table 21 3A) whose apparent glycine incorporation into uric acid was 11 per cent in 7 days might in reality have incorporated 0.40 per cent if (1) his miscible pool were twice normal and (2) his uricolysis (i.e. intestinal destruction of urate) were one-third greater than normal. These are not unreasonable expectations for a gouty patient with a serum uric acid level of 10.6 mg per 100 ml as reported for L. O. [199]

A second possible explanation for normal incorporation values in some gouty subjects is that the incorporation of glycine may be an inconstant process. One patient has already been cited in whom successive glycine-N<sup>15</sup> studies gave variable results [198]. In addition, subject O. N. (Table 21 3 1) was studied on two occasions and showed different results each time. An initial study showed an abnormal value of 0.30 per cent in 5 days but when the patient was restudied about one year later he was found to incorporate only 0.17 per cent of a tracer dose of glycine 1 C<sup>14</sup>.

A word of explanation is also in order regarding the extraordinary incorporation values found in certain patients of whom W. R. a mildly gouty subject with a serum uric acid value of 6.4 mg per 100 ml and a urinary excretion of only 313 ± 61 mg per day [200] is perhaps the best example. The results underscore a disparity encountered occasionally

TABLE 21-31 INCORPORATION OF ORAL TRACER DOSE OF GLYCINE 1 C<sup>14</sup> INTO URINARY URIC ACID IN PATIENTS WITH PRIMARY GOUT

| Clinical status                   | Subject | Uric acid          |                              | Cumulative recovery of C <sup>14</sup> in urinary uric acid in percentage of administered C <sup>14</sup> |               | Reference |
|-----------------------------------|---------|--------------------|------------------------------|---|---------------|-----------|
|                                   |         | Serum<br>mg/100 ml | Urine<br>(basal)<br>mg/24 hr | 4-5 days  | 7 days        |           |
|                                   |         |                    |                              |   |               |           |
| Control                           | CL      | 4.4                | 350                          | 0.11  | 0.18          | [200]     |
|                                   | WW      | 4.4                | 346                          | 0.09  | 0.15          | [200]     |
|                                   | HB      | 4.2                | 320                          |   | 0.22          | [98]      |
|                                   | DI      | 4.6                | 455                          |   | 0.21          | [98]      |
|                                   | BA      | 4.7                |                              |   | 0.16          | [199]     |
|                                   | MM      | 4.9                |                              |   | 0.13          | [199]     |
| Gout (normal uric acid excretion) | TR      | 5.4                |                              |   | 0.11          | [199]     |
|                                   | ON      | 8.5                | 435                          | 0.30  | 0.39          | [200]     |
|                                   | DW      | 8.3                | 317                          | 0.31  | 0.40          | [200]     |
|                                   | WR      | 6.4                | 313                          | 0.61  |               | [200]     |
|                                   | VL      | 8.4                | 458                          | 0.23  | 0.38 (6 days) | [200]     |
|                                   | FB      | 7.4                | 455                          | 0.11  |               | [9]       |
|                                   | RR      | 7.2                | 460                          | 0.14  |               | [9]       |
|                                   | RW      | 7.1                | 463                          |   | 0.33          | [8]       |
|                                   | CMC     | 5.8-8.0            | 405                          |   | 0.20          | [98]      |
|                                   | ON      | 6.6                | 334                          | 0.17  |               | [98]      |
|                                   | RMcJ    | 9.0                | 513                          |   | 0.20          | [98]      |
|                                   | EH      | 7.9                | 514                          |   | 0.36          | [9]       |
|                                   | IS      | 10.0               | 383                          |   | 0.14          | [199]     |
|                                   | LO      | 10.6               | 368                          |   | 0.15          | [199]     |
| Gout (high uric acid excretion)   | LW      | 7.4                | 203                          |   | 0.09          | [199]     |
|                                   | EHe     | 8.8-4.5            | 1004                         |   | 0.51          | [200]     |
|                                   | DR      | 10.3               | 689                          |   | 0.73          | [199]     |
|                                   | GMI     | 8.7                | 587                          |   | 0.20          | [98]      |
| Asymptomatic hyperuricemia        | HH      | 8.0                | 680                          | 0.13  |               | [200]     |
|                                   | RA      | 7.8                | 343                          |   | 0.21          | [90]      |
| Urate renal lithiasis             | LHM     | 6.1                | 430                          | 0.22  |               | [98]      |
|                                   | TJB     | 8.1                | 1157                         |   | 0.11          | [98]      |

Excretion data on patients from Reference 28 published through the courtesy of Dr J. E. Seegmiller (personal communication).

conditions. It may be concluded that overincorporation of glycine 1 C<sup>14</sup> into urinary uric acid is a constant finding in hyperuricemic subjects who excrete excessive quantities of uric acid in urine and a frequent though not invariable finding in hyperuricemic subjects who excrete normal quantities of uric acid. These data would seem to imply that overproduction of uric acid from glycine and other small molecular weight precursors is present in a large percentage of gouty subjects including many of those with a normal urinary uric acid excretion. When one com-

divergent results in these three patients thus appear to parallel the variable results obtained with labeled glycine

Studies with Aminoimidazolecarboxamide Seegmiller Laster and Stetten have conducted extensive studies with the purine precursor 4 amino-5-imidazolecarboxamide labeled in C4 with either C<sup>13</sup> or C<sup>14</sup>. In normal man approximately 20 per cent of administered AIC-C<sup>13</sup> is excreted in urine as uric acid in 14 days [202]. The data show a biphasic

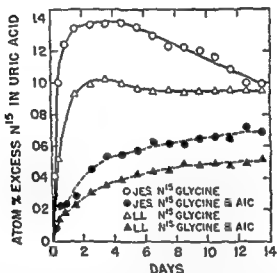


Fig 21-14 Concentration of  $N^{15}$  in urinary uric acid in two normal subjects receiving glycine  $N^{15}$  with and without AIC. Note that the concomitant administration of AIC greatly reduced the utilization of glycine  $N^{15}$  for uric acid synthesis (From J. E. Seegmiller et al. [10] with permission of authors and publisher.)

incorporation. There is a prompt and extensive conversion of ingested AIC-C<sup>13</sup> into uric acid followed by a slower, less direct conversion process. In all of 5 gouty subjects studied [206-207], incorporation of AIC into uric acid was somewhat greater than normal irrespective of urinary urate excretion. The degree of abnormality was magnified when appropriate corrections were made for dilution factors within the urate pool and for uricolyis on the basis of simultaneous studies with uric acid-C<sup>14</sup>.

In addition, these investigators studied simultaneously the incorporation of glycine- $N^{15}$  into urinary uric acid [202]. Administration of AIC (in dosage of 0.1 gm per kg) significantly suppressed incorporation of  $N^{15}$  into uric acid in both normal (Fig. 21-14) and gouty subjects but frequently less effectively in gouty persons. These results have important implications for the fundamental nature of the gouty defect as will be seen in a later section.

between incorporation values and urinary excretion. The factors of concurrent tophus deposition and extrarenal disposal are partial but not adequate explanations for these results. A more plausible one is that early incorporation of glycine into uric acid and total production of uric acid do not always bear a *pari passu* relationship one to another. A possible explanation of this dissociation based on a variable contribution of the shunt or direct pathway of urate synthesis (see below) to total synthesis has previously been offered [200].

Nevertheless despite interpretative difficulties presented by individual studies the data shown in Table 21-3-4 do provide evidence that glycine incorporation and uric acid production are correlated. A plot of the  $C^{14}$  incorporation values (6 to 7 days) against the urinary excretion of uric acid including all subjects (control gout urolithiasis) shows a positive correlation having a high degree of statistical significance ( $r = 0.7476$ ,  $t = 4.906$ ,  $p = < 0.01$ ). This result provides considerable warrant for regarding the glycine-1  $C^{14}$  incorporation data as generally reflecting closely the production of uric acid and for interpreting overincorporation of this tracer into urinary uric acid as indicative of overproduction.

Studies have also been performed with glycine-2  $C^{14}$  in a small number of subjects [199] but these suffer from a limitation that also applies to glycine- $N^{15}$  viz, the occurrence of secondary labeling of several atoms of the purine ring other than the one for which the labeled atom is a specific precursor. In the case of glycine-2  $C^{14}$  labeling of carbon 2, -4, -6 and -8 occurs in addition to that of carbon 5 which is specifically donated by the  $\alpha$  carbon of glycine [72, 201]. In the case of glycine- $N^{15}$  isotope is found in nitrogen atoms other than N7 in appreciable concentration and increasingly so with time [70, 202]. In contrast glycine-1  $C^{14}$  labels C4 of urinary uric acid specifically [71, 72, 200]. Glycine-1  $C^{14}$  has another advantage over glycine- $N^{15}$  as a precursor for human studies in that it can be given in a tracer dose. The dose of glycine- $N^{15}$  required 50 to 100 mg per kg is of the order of magnitude of the miscible glycine pool itself [199, 203] and such loading doses result in two- to tenfold reductions in the percentage of isotope entering uric acid [199, 200]. For these reasons studies with tracer doses of glycine-1  $C^{14}$  have yielded the most reliable information regarding uric acid generation in hyperuricemic subjects.

A limited number of studies of rate of generation of urate has also been made with formate  $C^{14}$  as the test substance. In two gouty subjects who were excreting 830 and 950 mg uric acid daily Spilman [204, 205] found that both the rate and magnitude of labeling of urinary uric acid were excessive in comparison with two control subjects. Subsequently Villa Robecchi and Ballabio [164] found no evidence of increased incorporation of formate  $C^{14}$  into urinary uric acid in one patient with chronic tophaceous gout and a urinary urate excretion of only 420 mg per day when results were compared with those in one control subject. The

suggested that IMP was not the only nucleotide subject to cleavage shortly after formation

In three gouty subjects patterns were obtained in urinary purines following administration of glycine  $1\text{ C}^{14}$  which were similar to those in control subjects but there were two potentially important differences (Fig 21 16) Initial hypoxanthine labeling values were never higher than

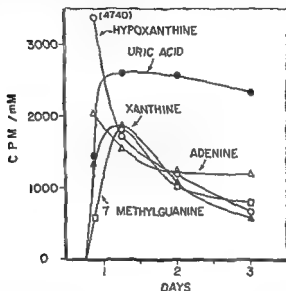


Fig 21 15 Specific activity values of urinary purines in a normal subject following oral administration of glycine  $1\text{ C}^{14}$  (From J Clin Invest 37 519 1958 with permission of the publishers)

those of corresponding uric acid samples and a striking initial labeling of 7 methylguanine was found. These results suggested that in gouty subjects the shunt pathway involved a more rapid turnover of the hypoxanthine pool than in normal subjects and that guanine nucleotides may have been of greater importance as intermediates than in normal persons. Since the initial labeling pattern in a patient with myeloid metaplasia resembled that of patients with primary gout especially with reference to labeling of hypoxanthine and 7 methylguanine the pattern in gouty patients was also considered a consequence of overproduction of purine nucleotides and not necessarily indicative of a fundamental difference from normal in pathways available for rapid uric acid synthesis.

A study [214] with labeled hypoxanthine demonstrated rapid and extensive conversion to uric acid. A study with labeled adenine indicated that free adenine was not a significant participant of the shunt pathway in that it was but slowly and sparingly converted to uric acid probably

**The Shunt Pathway(s) of Uric Acid Synthesis** In the initial studies of the generation of uric acid from glycine  $N^{15}$  the rapidity and extent of overincorporation of isotope into urinary uric acid in gouty hyperexcretors led Stetten [208] to postulate a shunt pathway by which dietary glycine nitrogen could enter the purine nucleus more promptly than in normal man by passing nucleic acid purines whose turnover appeared to be too slow to account for these results [209]. The same reasoning may be employed however to postulate that all subjects possess such a rapid pathway for uric acid synthesis since significant labeling of uric acid occurs within hours in normal and in mildly gouty subjects as well as in severely gouty subjects. This is true regardless of the precursor employed although best illustrated following administration of AIC. The prompt and excessive incorporation of precursors into uric acid in the gouty subject might be explained either by an accentuation of the normal shunt pathway or by the operation of a new perhaps abnormal pathway also by passing nucleic acids.

Stetten [210] drew an interesting analogy between the gouty subject and the bird which is known to excrete waste nitrogen as uric acid. It does so by a pathway involving rapid synthesis of IMP followed by cleavage reactions yielding hypoxanthine [211] which is readily oxidized to uric acid. Stetten suggested this pathway as one possibility to explain rapid and excessive incorporation of glycine- $N^{15}$  into urinary uric acid in certain gouty subjects. It would suffice equally well to explain the prompt labeling observed in normal subjects. Goodwin [212] has devised a technique for evaluating the magnitude of this shunt pathway based on simultaneous administration of glycine  $1\text{-C}^{14}$  and formate- $\text{C}^{14}$  and on comparisons of rates of turnover of precursor pools with rates of appearance of isotope in appropriate atoms of urinary uric acid. In one normal subject the shunt pathway accounted for synthesis of 70 mg uric acid per day. Studies on gouty subjects should prove of great interest.

An attempt has been made to study possible intermediates of the shunt pathway in normal and gouty subjects indirectly by measurement of labeling of various urinary purine bases following administration of glycine  $1\text{-C}^{14}$ , AIC  $4\text{-C}^{14}$ , hypoxanthine  $8\text{-C}^{14}$  or adenine  $8\text{-C}^{14}$ . Since purine bases do not arise *de novo* but rather by cleavage of the corresponding nucleotide inferences were drawn regarding possible nucleotide intermediates of the shunt pathway.

In two normal subjects given glycine  $1\text{-C}^{14}$  a very prompt and striking labeling of urinary hypoxanthine resulted and specific activity curves of hypoxanthine and uric acid bore a precursor-product relationship [213] (Fig. 21-15). These results constitute strong evidence for the operation of the IMP cleavage pathway in normal man. In addition the early labeling of such urinary purine bases as adenine, 7-methylguanine and xanthine (believed to be derived chiefly from guanine in most subjects)

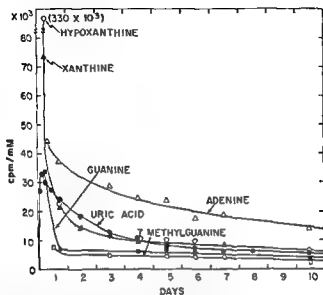


Fig 21-17 Specific activity values of urinary purines in a patient with chronic myelogenous leukemia following intravenous administration of  $^{14}\text{C}$  (From *Metabolism* 8:455-1959 with permission of the publisher)

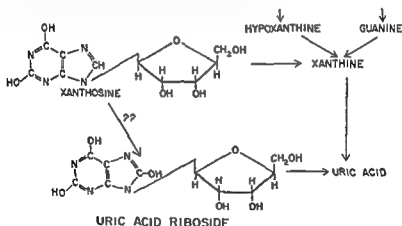


Fig 21-18 Utilization and disposal of purine

from this source and one would have to know the ratios of  $\text{N}^{15}$  and  $\text{C}^{14}$  in the precursor glycine pool as functions of time in order to interpret the data presented. At present therefore an accessory pathway for uric acid formation is speculative but the results presented by Easter and Cutman and their coworkers warrant further study.



via more complex ribosyl intermediates. I labeled AIC on the other hand gave rise to prompt and extensive labeling of hypoxanthine, xanthine, and uric acid and to a lesser extent also of adenine, guanine and 7-methylguanine (Fig. 21-17). These findings strengthened the concept that IMP cleavage was the major but by no means the sole pathway contributing to rapid synthesis of uric acid in normal and gouty man.

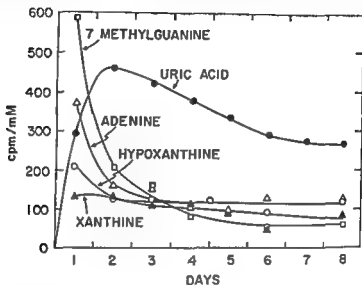


Fig. 21-16. Specific activity values of urinary purines in a gouty subject following oral administration of glycine-1- $C^{14}$  (from *J. Clin. Invest.* 37:513, 1958, with permission of the publishers).

The possibility that accessory pathways of uric acid synthesis exist which differ from conventional pathways of nucleotide cleavage and purine base oxidation may also be considered [215]. Cleavage of uric acid ribonucleoside [153] would offer a pathway for synthesis of uric acid not involving xanthine as an immediate precursor. Such a pathway would accord nicely with isotopic labeling studies of urinary purines in which hypoxanthine and uric acid were highly labeled but xanthine was not. A possible route of synthesis of uric acid ribonucleoside is by direct oxidation of xanthosine (Fig. 21-18) but this is not established. The role of the ribonucleoside in uric acid synthesis by normal or gouty man remains to be determined.

A second possibility regarding accessory pathways of uric acid synthesis has been raised by Gutman and associates [199] on the basis of ratios of ( $N^{15}$  in  $N_7$ ) to (total  $C^{14}$  in uric acid) in excess of 1 in studies in which glycine- $N^{15}$  and glycine-1- $C^{14}$  were administered simultaneously. However, since glycine  $N^{15}$  contributes  $N^{15}$  to the general nitrogen pool, the newly synthesized glycine reentering the glycine pool may derive  $N^{15}$

# TUBULAR REABSORPTION OF URIC ACID

The tubular transport mechanisms for reabsorption of urate though not identified are presumed to be enzymatic. Berliner et al [229] have shown that net tubular reabsorption is characterized by a limiting maximal rate of the order of 15 mg per min per 1.73 m<sup>2</sup>. The magnitude of the  $T_m$  is such however that it is almost certainly never exceeded under physiologic conditions since at a normal filtration rate of 120 ml per min saturation would not occur below plasma levels of 15.8 to 18.7 mg per 100 ml. In patients with depressed filtration rates but normal tubular function even higher values would be required.

The normal net clearance of urate is of the order of 7 to 10 ml per min, a quantity equal to 5 to 10 per cent of the filtered urate load. Normally appears in urine [230-231] and this figure is substantially maintained even in the presence of a markedly reduced glomerular filtration rate unless widespread tubular disease is present.

In studies of renal clearance of urate a dependency upon rates of urine formation has been observed. At urine flow rates below 1 ml per min apparent tubular reabsorption is quantitatively greater than at higher flow rates [231]. Beyond 1 to 2 ml per min no further augmentation of clearance occurs [164].

## RENAL EXCRETION OF URIC ACID IN GOUT

A. H. Garrod first postulated in 1859 that an inherited renal disability in the excretion of uric acid was the cause of the hyperuricemia of gout. Numerous later workers accepted and defended this explanation for the hyperuricemia of the disease [232]. This conclusion was based on the observation that urinary urate excretion in gouty individuals was generally within the normal range and occasionally even low despite considerable elevation of blood or serum urate levels.

With the advent of the clearance concept and the development of techniques for study of discrete renal functions it became possible to evaluate the renal handling of urate by the normal and gouty kidney. An impressive number of studies has now been published on this topic. Many of these have been reviewed by Gutman and Yu [233] in their notable paper on renal function in gout.

Gutman and Yu conducted studies of renal function in some 300 gouty patients and standard renal clearance procedures on approximately 100 cases including some asymptomatic hyperuricemic relatives of these gouty subjects. In general glomerular filtration rates ( $C_{cr}$ ), renal plasma flow ( $C_{in}$ ) and maximal tubular excretory capacity ( $T_{m, ur}$ ) corresponded with those of normal subjects of equivalent age. However impairment of renal hemodynamics (particularly  $C_{PAH}$ ) was not infrequent especially in elderly patients and in those with overt renal dis-

## RENAL HANDLING OF URATE

## TUBULAR SECRETION OF URIC ACID

There is now good evidence for tubular secretion of urate in man and although it has been demonstrated only under quite unphysiologic conditions its very demonstration requires that existing notions regarding urate excretion in normal and gouty man be carefully reevaluated. Although tubular secretion of urate was suggested by early workers under the influence of the Cushny school the mechanism of renal handling of urate came to be regarded as exclusively one of glomerular filtration followed by partial tubular reabsorption. Because plasma urate is essentially unbound and freely ultrafiltrable complete glomerular filtration has been assumed. Existing clearance data have been interpreted as indicating prompt tubular reabsorption of all but 5 to 10 per cent of the filtered urate load. The data are equally consistent with quantitative tubular reabsorption of filtered urate followed by tubular secretion of a quantity of urate equivalent to 5 to 10 per cent of the filtered load. Finally, they could represent the net result of incomplete tubular reabsorption and tubular secretion. The present dilemma renders hazardous too positive a treatment of available data.

The occurrence of tubular secretion of urate was demonstrated by Gutman Yu and Berger [216] in subjects who were given a potent uricosuric drug and were rendered markedly hyperuricemic by infusions of lithium urate while subjected to pronounced osmotic diuresis with mannitol. In several subjects  $C_{ur}$  to  $C_{cr}$  values and excreted urate to filtered urate ratios considerably in excess of 1.0 were achieved. These studies impart a new plausibility to an earlier report [217] of  $C_{ur}$  to  $C_{cr}$  values ranging from 1.28 to 1.46 in a young man with defective tubular reabsorption of uric acid and indicate that among mammals man as well as the rabbit [218] and the Dalmatian coach hound [219] possess a mechanism for tubular secretion of urate.

The demonstration of tubular secretion of urate in man was a logical goal since many observations are difficult to explain on the filtration reabsorption theory alone. These include the striking dissociation of filtered urate values and urinary urate excretions [220] the paradoxical effects of uricosuric agents which cause retention of urate when given in small doses [221-222] the profound reduction in renal clearance of urate caused by such substances as pyrazinoic acid [223] pyrazinamide [224-225] chlorothiazide [226] and lactate [227] and the hyperuricemia of toxemia of pregnancy which is greater than can be attributed to glomerular functional changes alone [229]. In all these situations variation or inhibition of tubular secretion of urates provides a less awkward interpretation than that of enhancement of tubular reabsorption.

gouty patients with normal daily uric acid excretions or those with elevated daily uric acid excretions showed statistically significant difference from control subjects in the percentage of filtered uric acid excreted in urine. This difference could be viewed as indicating a more effective tubular reabsorption or less active tubular secretion of uric acid or both.

TABLE 21-4A NET TUBULAR REABSORPTION OF URIC ACID IN PRIMARY GOUT

| Controls |            | Subjects with gout |            | Difference<br>per cent | Significance | Reference |
|----------|------------|--------------------|------------|------------------------|--------------|-----------|
| No       | Per cent   | No                 | Per cent   |                        |              |           |
| 61       | 91.9 ± 2.3 | 150                | 93.1 ± 2.0 | 1.2                    | $p < 0.005$  | [35, 38]  |
| 13       | 0.1        | 9                  | 9.6        | 3.5                    | $p < 0.001$  | [240]     |
|          | 91.7       | 56                 | 9.8        | 4.1                    | $p < 0.01$   | [104]     |

Values given are percentages of the filtered uric acid load reabsorbed in the renal tubule.

TABLE 21-4B NET EXCRETION OF URIC ACID IN PRIMARY GOUT

| Controls |           | Subjects with gout                                    |     |           | Difference<br>per cent | Significance      |
|----------|-----------|---|-----|-----------|------------------------|-------------------|
| No       | Per cent  | Type  | No  | Per cent  |                        |                   |
| 61       | 7.0 ± 2.4 | A All subjects  | 150 | 6.8 ± 2.1 | 0.8                    | $0.05 > p > 0.01$ |
| 61       | 7.6 ± 2.4 | B Hyperexcretors<br>with GFR > 100                    | 40  | 6.0 ± 2.0 | 1.0                    | $0.05 > p > 0.0$  |
| 61       | 7.6 ± 2.4 | C Normoexcretors<br>with GFR > 100                    | 54  | 5.9 ± 1.7 | 1.7                    | $p < 0.01$        |
| 61       | 7.6 ± 2.4 | D Asymptomatic<br>hyperuricemic<br>subjects<br>B vs C | 11  | 5.5 ± 0.4 | 2.1                    | $p < 0.01$        |
|          |           |   |     |           | 0                      | $0.1 > p > 0.05$  |

Values given are percentages of the filtered uric acid load excreted in the urine.

Source: Recalculated from A. H. Cuthbert et al. [33].

Recently Nugent and Tyler [241] have published similar data on control and gouty subjects and have shown in addition that nongouty males respond to an increased plasma uric acid concentration with an increase in clearance of uric acid so that at equivalent serum uric acid levels the disparity in  $C_{\text{crea}}$  to  $C_{\text{ura}}$  ratios between nongouty and gouty subjects is further enhanced. Bishop [238] has suggested that the kidney in gout may be limited in its ability to respond to an elevation of plasma uric acid level with an increase in uric acid clearance such as appears to occur in nongouty subjects. Critical studies of this point will be of great interest. To date the studies on the two groups are not strictly

ease. The 24 hr urinary urate excretion was *within* the normal range (mean  $\pm$  s.d. =  $418 \pm 70$  mg per 24 hr, range = 278 to 538 mg per 24 hr) in 67.0 per cent of patients whereas in 4.3 per cent it was *below* and in 28.7 per cent it was *above* the normal range. In 18.0 per cent the values exceeded the normal mean by more than 3 s.d. The filtered urate load, calculated as the product of the plasma urate level and  $C_{cr}$ , was found to be increased in most gouty subjects and this was regarded as further evidence that the metabolic defect in gout was prerenal [230].

To this point the studies are in agreement with earlier studies of the same problem [234-237]. Of special note are those of Talbott and Coombs [237] and of Friedman and Byers [236] on young gouty subjects without evidence of renal insufficiency. Both groups have emphasized that these subjects had normal urate clearances and excreted greater than normal quantities of urate in urine. A number of the patients studied by Gutman and Yu showed similar findings. These observations are highly significant for they cannot be explained on the basis of the Garrod theory of hyperuricemia and strongly suggest that hyperuricemia is a consequence of overproduction of uric acid in these patients. These data tacitly suggest that the finding of a normal or low value of urinary uric acid may be related to the duration of hyperuricemia. To some extent this may be true since impairment of renal hemodynamics increases with age. However, not all young gouty patients show normal urate clearances. The author is presently studying two young gouty subjects, ages 14 and 21, without significant renal functional abnormalities, whose serum urate levels range from 10 to 12 mg per 100 ml and whose urinary urate values range from 260 to 290 mg per 24 hr. The younger patient has had sore heels for 6 months and had a normal serum urate level  $1\frac{1}{2}$  years prior to his first symptom. Existing data in both subjects suggest defective renal excretion of uric acid.

The interpretation of the significance of values for presumptive tubular reabsorption of urate is now difficult. In the study by Gutman and Yu the mean net tubular reabsorption was 91.9 per cent of the filtered urate in normal subjects and 93.1 per cent in the gouty subjects. The distributions of the two groups overlapped almost wholly. Nevertheless the difference in means is highly significant as Bishop has pointed out [238]. In agreement with this reappraisal, certain European investigators have reported greater net tubular reabsorption of urate in gouty subjects than in controls [164, 239, 240]. These data are summarized in Table 21-4A. In Table 21-4B the data of Gutman and Yu have been recalculated and are presented in terms of net excretion of uric acid. Only those gouty subjects with glomerular filtration rates of 100 ml per min ( $C_{cr}$ ) have been included in order to eliminate in so far as possible gross changes perhaps attributable to renal insufficiency. All groups of gouty subjects, whether asymptomatic hyperuricemic patients, clinically

studies with labeled and unlabeled uric acid both indicate that a significant quantity of uric acid is disposed of by routes other than the kidney.

In initial studies with uric acid  $N^{15}$  small though significant concentrations of  $N^{15}$  were found in urinary urea and ammonia [188]. Subsequently when a relatively large quantity of uric acid  $N^{15}$  was administered intravenously to a normal subject [189] highly significant concentrations of isotope were found in urinary urea and ammonia proving

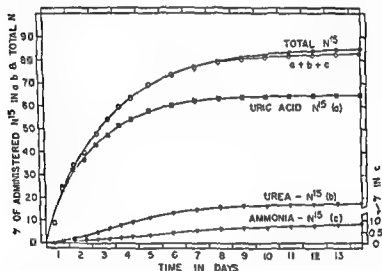


FIG 21 19 Cumulative recoveries of  $N^{15}$  in urinary nitrogenous constituents following intravenous administration of uric acid  $13-N^{15}$  to a normal subject (From *J Biol Chem* 203 9 1953 with permission of the publishers)

the occurrence of uricoly is. After 2 weeks about 17 per cent of the injected  $N^{15}$  was recovered as urea and about 1 per cent as ammonia (Fig 21 19). Following intravenous injection of uric acid  $N^{15}$  from 6 to 19 per cent of administered  $N^{15}$  was recovered in fecal nitrogen [189 245]. In patients with biliary catheters given uric acid  $N^{15}$  labeled products were recovered in bile. The results suggested that uricoly is occurred in the intestinal tract but initial attempts to inhibit uricoly is by administration of oral sulfonamide were unsuccessful [189].

Recently Sorenson [184] has repeated the studies using uric acid  $2-C^{14}$ . After intravenous administration  $C^{14}$  was found in respiratory  $CO_2$ , urinary urea, allantoin and allantoic acid and feces (Table 21 5). The total recovery in degradation products was 22.5 per cent with uric acid  $N^{15}$  the comparable value was 25.8 per cent [189]. However an interesting difference appeared in the two studies. With the passage of

comparable as they have dealt only with nongouty subjects in whom plasma uric acid has been varied relatively acutely and with gouty subjects after years of sustained hyperuricemia

Nugent and Tyler [251] conclude that abnormal renal excretion of uric acid is one important cause of hyperuricemia in some patients with gout. It is in all probability at least a major contributing factor but the renal factor fails to offer a satisfactory explanation for the observation that a highly significant minority of gouty patients excrete excessive quantities of urate, an observation that can be explained only by overproduction of uric acid. One might intuit that there are two (or more) heterogeneous types of gout: one in which hyperuricemia is attributable to abnormal renal excretion of uric acid; another in which it is attributable to overproduction of uric acid. However, the fact that the group of gouty subjects with excessive urinary urate excretion and the group of gouty subjects with normal uric acid excretion both show a low net urate clearance fails to provide a sound basis for this division. These data suggest that abnormal renal excretion of uric acid may be characteristic of primary gout regardless of its stage or of the level of urinary urate but they do not indicate that the renal factor is necessarily the fundamental gouty defect. Is it possible that the gouty individual produces in abnormal quantity some substance which has an effect on renal excretion of urate similar to that of lactate? Such an explanation might harmonize the overproduction/underexcretion conflict for both might be operating simultaneously.

### URICOLYSIS

In a sense gout may be considered a consequence of the genetic deletion of uricase in man for if uricase were present there would be no hyperuricemia and presumably no gout. Despite the absence of uricase in human tissues [242, 243] uric acid is not the exclusive end product of purine metabolism in man. A variety of studies has shown that uric acid is subject to limited catabolism in the human being.

Urinary recoveries of injected uric acid are incomplete in normal subjects. Thirty-five years ago Folin, Berglund, and Derick [63] and Koehler [244] reported recoveries which ranged from 28 to 91 per cent and averaged about 50 per cent. More recently recoveries ranging from 55 to 95 per cent of uric acid  $N^{15}$  or  $C^{14}$  have been reported [184, 185, 189, 191, 193, 246]. The average of 14 studies with uric acid  $N^{15}$  was 75  $\pm$  per cent.

Studies of turnover of uric acid in normal man have uniformly shown that the quantity of uric acid synthesized per day is greater than the quantity appearing in urine [184, 188]. In studies with uric acid  $N^{15}$  [185] or  $C^{14}$  [184] the fraction of the turnover appearing in urine is essentially the same as the fraction of injected uric acid recovered in urine. Thus

are shown in Table 21 ■ where it may be seen that the quantity of  $C^{14}$  recovered in various degradation products was reduced from 22.5 to 3.0 per cent during drug treatment. Moreover during intestinal bacteriostasis uric acid was found in feces whereas none was found in the initial experiment. An average of 186 mg per day of uric acid or 26.6 per cent of the total turnover was excreted in feces over a 5 day period during bacteriostasis a figure agreeing well with the recovery of 30.1 per cent of administered  $C^{14}$  in stools. There can no longer be much doubt that intestinal bacterial degradation is the major cause of uricolysis in man. A variety of intestinal organisms have the capacity to destroy uric acid particularly *Aerobacter aerogenes* which may constitute 5 per cent or more of the intestinal coliform population [189].

Sorensen [184] also reinvestigated the content of uric acid in saliva, gastric juice and bile and estimated that 100 mg or more enters the alimentary tract each day in these juices. An equal quantity may also enter in pancreatic and intestinal juices. The quantities of uric acid entering the intestinal tract therefore are larger than previously estimated [248-249] and are adequate to account for degradation of one third of the uric acid normally turned over each day.

There remains the question of uricolysis within tissues of man. Two enzyme systems of human tissues can destroy uric acid in vitro at physiologic pH viz. peroxidase [250] and cytochrome cytochrome oxidase [251]. Additionally Bien and Zucker [252] have shown that leukocytes and erythrocytes of normal subjects will destroy uric acid during prolonged incubations and Villa Polli and Busc [252a] have demonstrated uricolytic properties of leukocyte extracts. This activity resides primarily in cells of the myeloid series [253] which are known to contain peroxidase [254]. Cancalakis, Tuttle and Cohen [250] have shown that the products of peroxidative destruction of uric acid in phosphate buffer are chiefly urea and allantoin. In a recent study of the mechanism of peroxidatic uricolysis Howell has found that the apoenzyme is unnecessary and that the prosthetic group hematin actively destroys uric acid in the presence of hydrogen peroxide yielding allantoin and other products [255].

In Sorensen's experiments [184] the excretion of  $C^{14}$  in urea and allantoin was relatively little changed during intestinal bacteriostasis despite profound alterations in the amounts of  $C^{14}$  found in other degradation products (Table 21-5). This suggested that allantoin and possibly some of the urea were not derived from intestinal breakdown of uric acid. The common denominator of mammalian systems capable of destroying uric acid is the presence of a hematin prosthetic group. Since a small but detectable steady state concentration of hydrogen peroxide exists in living tissue (Chap. 46) it may be that a nonenzymatic hematin peroxidatic destruction of uric acid proceeds to a limited extent in human tissue.



time following injection of isotopic uric acid an increasing fraction of  $N^{15}$  was found in urinary compounds other than uric acid, reaching 50 per cent on the tenth day whereas the amount of  $C^{14}$  in urinary urea averaged only 2.5 per cent of  $C^{14}$  in uric acid throughout the experiment. This difference is probably attributable to late recycling of  $N^{15}$  into urinary urea, a process minimized with  $C^{14}$  in which respiratory  $CO_2$  constitutes the chief breakdown product.  $CO_2$  accounts for 10 to 16 per cent of  $C^{14}$  administered as uric acid, 2  $C^{14}$  in normal man [184, 246].

TABLE 21.5 RECOVERY OF INTRAVENOUSLY ADMINISTERED URIC ACID 2  $C^{14}$  IN EXCRETORY PRODUCTS (5-10 DAYS) BEFORE AND AFTER ESTABLISHMENT OF EFFECTIVE BACTERIOSTASIS OF THE INTESTINAL TRACT

| Excretory product                      | Recovery of $C^{14}$ (per cent of dose) |                       |
|--|---|-----------------------|
|  | Before bacteriostasis                   | During bacteriostasis |
| Urinary uric acid                      | 63.0 (10 days)                          | 50.7 (5 days)         |
| Urinary allantoin                      | 2.1                                     | 1.8                   |
| Urinary allantoic acid                 | 0.2                                     |                       |
| Urinary urea                           | 2.2                                     | 0.7                   |
| Expired carbon dioxide                 | 10.9                                    | 0.5                   |
| Fecal products                         | 7.1                                     | 0.0                   |
| Total recovery in degradation products | 22.5                                    | 3.0                   |

SOURCE: L. H. Sorensen [184].

In experiments with uric acid  $N^{15}$  about 2 per cent of  $N^{15}$  appeared in urinary allantoin [247]. Following administration of uric acid 2  $C^{14}$  from 0.4 to 4.5 per cent of  $C^{14}$  appeared in urinary allantoin and comparable amounts were recovered in urinary allantoic acid [184].

### THE SITES OF URICOLYSIS

When labeled uric acid was administered orally to normal subjects only 0 to 11 per cent was absorbed and excreted unchanged in urine [184, 191]. With uric acid  $N^{15}$  47 per cent of the  $N^{15}$  was recovered in urinary urea in 3 days [191]. With uric acid 2  $C^{14}$  only 2.4 per cent appeared in urea in 5 days [184]. Fifty-five per cent of the  $C^{14}$  was excreted as respiratory  $CO_2$  in 2 days. An additional 16.3 per cent was recovered in feces. 83 to 91 per cent of this amount being found within the intestinal bacteria themselves. Only a trivial amount was found in fecal uric acid. These findings implicated intestinal bacteria as the agents responsible for the breakdown of the orally administered uric acid.

To verify the role of the intestinal flora in uricolysis in man the degradation of intravenously administered uric acid was studied in a normal subject before and after an effective bacteriostasis was achieved with concomitant sulfonamide, streptomycin and neomycin [184]. The results

Secondary gout occurs as a complication of certain disorders of the hematopoietic system in which, as a consequence of accelerated turnover of nucleic acids there is an overproduction of uric acid and other purines. Talbott [256a] and Gutman and associates have recently reviewed their experiences in this disorder [257-259]. It occurs with some frequency in myeloid metaplasia [258] in 5 to 9 per cent or more of patients with polycythemia vera (especially in those cases merging into the phase of myeloid metaplasia) [260-261] occasionally in secondary polycythemia [262] chronic myelogenous leukemia acute leukemia pernicious anemia Cooley's anemia other chronic hemolytic anemias in adults [263] and multiple myeloma [264]. The greatest incidence is in those disorders grouped by Dameshek [265] as the myeloproliferative syndromes.

The common denominator of these disorders is hyperuricemia consequent to increased formation and degradation of nucleic acids. The term secondary gout is presently reserved for those patients who develop clinical manifestations of gout acute or chronic responsive to the usual therapeutic measures. This restriction in the use of the term is not altogether satisfactory for the predilection toward development of clinical gout may be present even in hyperuricemic individuals of this group who never develop symptoms of gout just as it is presumed to exist in hyperuricemic members of gouty families all of whom are believed to carry the gouty trait.

In many cases clinical gout in a patient with a myeloproliferative disorder may represent chance concurrence of primary gout with a second relatively common disease. There are a number of differences between primary and secondary gout however which suggest that secondary gout occurs as an acquired disorder. Its incidence in polycythemia vera 2 to 10 per cent is too high to be due to chance. The incidence in females in the study by Lu and Gutman of 20 cases [259] was much higher (30 per cent) than in 540 cases of primary gout (3 per cent) and the mean age of onset of overt gout was much higher (54 vs 40 years). Moreover there may be a distinct temporal relationship to therapy such as radiation or chemotherapy in polycythemia or leukemia or administration of liver or vitamin B<sub>12</sub> in pernicious anemia. And finally familial occurrence is not conspicuous [257].

The serum level of uric acid in patients with secondary gout tends to be higher than in those with primary gout. The mean value in the series of Lu and Gutman [259] was 12.0 mg per 100 ml compared with a mean of 9.1 mg per 100 ml in primary gout. The mean urinary excretion of uric acid was also greater 631 compared with 497 mg per day. Excretion of uric acid varies quite widely among these patients depending in part upon the type of hematopoietic disturbance present [266].

Urinary excretion of purine bases has also been studied in selected patients with disorders of the type associated with secondary gout [267].

The extent of this reaction probably would not exceed 2 to 4 per cent of the uric acid turnover per day as a maximal value. The urinary allantoin values normally some 10 to 70 mg per day reflect in large part ingested allantoin [247]. On a 'purine free' diet (of necessity also allantoin poor) Wicelowski [256] found allantoin excretion to be not more than 11 mg per day. This value may approximate the endogenous allantoin production and is within the range of the turnover percentage suggested above.

### URICOLYSIS IN GOUT

Decreased uricolysis has been proposed as a cause of hyperuricemia in gout [63, 252]. However, recoveries of injected unlabeled uric acid have ranged from 15 to 94 per cent in the urine of gouty subjects [63, 244]. Recoveries of injected uric acid  $N^{15}$  [245] or  $C^{14}$  [184] have ranged from 34 to 54 per cent in urine. All these values are lower, on the average, than those found in nongouty subjects. Furthermore, in gouty subjects the fraction of the daily turnover of uric acid recovered in urine is smaller than in normal subjects. These studies are complicated by the presence of solid urate with which labeled urate may exchange [190], but evidence in other studies has also suggested that extra renal disposal of uric acid is greater than normal in gouty subjects [184]. Indeed, uricolysis should be accentuated in hyperuricemic individuals since in hyperuricemia a larger than normal quantity of uric acid would enter the intestinal tract. Polycove et al. [246] have reported a greater than normal yield of  $C^{14}O_2$  from injected uric acid  $C^{14}$  in hyperuricemic subjects.

Bien and Zucker [252] observed less destruction of uric acid by whole blood, white or red blood cells of gouty subjects than of normal persons during a 24 hr incubation study. For example, a mean decrease of  $34.4 \pm 3.2$  per cent was observed in plasma urate after 24 hr incubation of normal whole blood compared with  $23.2 \pm 4.2$  per cent in blood from gouty subjects. Since only slight uricolysis occurred during the initial 6-hr period, the destruction observed may have been due to factors not operating *in vivo*. The maximal uricolysis attributable to the tissues of man, 2 to 4 per cent of the uric acid turnover, is too small to be compatible with these results. Thus, no substantial evidence exists to implicate failure of uricolysis in the hyperuricemia of gout. On the contrary, emphasis should be placed upon enhanced enteric uricolysis as a compensatory factor in gout, tending to lessen hyperuricemia and perhaps constituting the major process of disposal of uric acid in patients with severe renal insufficiency.

### SECONDARY GOUT

A discussion of secondary gout is warranted in this chapter because of the light it may help throw on metabolic problems of primary gout.

In the patient with myeloid metaplasia given glycine  $1\text{ C}^{14}$  [218] the labeling of intermediary purines in urine clearly showed that processes of urate formation could be dissected into two classes—those yielding urate by rapid pathways manifested by maximal enrichment of precursor purines in a matter of hours and those yielding urate by lower pathways reflected in attainment of secondary maxima in precursor purines some days later (Fig. 21-20). The major difference between these

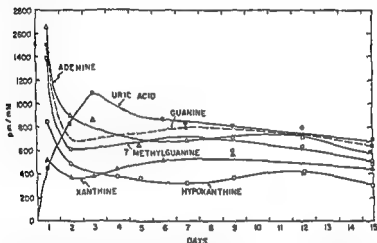


Fig. 21-20 Specific activity values of urinary purines in a patient with myeloid metaplasia following oral administration of glycine  $1\text{ C}^{14}$ . (From Metabolism 8:44, 1957 with permission of the publishers.)

patterns and ones obtained in normal subjects or patients with primary gout lay in the great quantitative accentuation of the slower pathway as evaluated by inspection of enrichment curves of precursor purines. In harmony with these results and interpretations are data presented by Weissmann, Bromberg, and Gutman [161] on enrichment of precursor purines in urine following administration of glycine- $\text{N}^{14}$  to a patient with polycythemia.

The data illustrate clearly that the predominant pathways of uric acid biosynthesis are different in primary and secondary gout. Patients with myeloproliferative disorders are distinguished from normal subjects and patients with primary gout by an accelerated and augmented turnover of nucleic acids. According to present concepts, however, a large portion of pathways involved in this derangement of purine metabolism is common also to normal persons and patients with primary gout, viz. the reactions generating purine nucleotides from small molecular weight precursors and those involved in nucleotide interconversions and breakdown and oxidation of bases to uric acid.

In general, these patients tend to show normal or low values of hypoxanthine, xanthine and 1 + 7 methylguanines and normal or high values of adenine, guanine 7-methyl 8 hydroxyguanine, and succinoadenine. In addition increases of urinary inosine and guanosine and of certain other purine and pyrimidine compounds have been noted in leukemic subjects [177].

The miscible pool of uric acid and its turnover have been determined in only a few subjects of this category. Both were increased in one leukemic and one of two polycythemic patients studied by Bishop Garner and Talbott [192]. In contrast a larger number of studies of purine production from labeled precursors has been carried out. The rate curves of glycine- $N^{15}$  incorporation into uric acid in primary and secondary polycythemia [262, 267] and in myeloid metaplasia [268] reveal a slowly progressive enrichment with maxima appearing from 10 to 14 days after glycine ingestion in contrast to the early peak found in normal subjects and patients with primary gout. Cumulative incorporation of  $N^{15}$  into uric acid was greater in patients with abnormalities of the myeloid series as in primary polycythemia and myeloid metaplasia than in patients with polycythemia secondary to congenital heart disease. The presence of secondary gout introduced no distinguishing features in the curves of glycine  $N^{15}$  enrichment. The late maxima observed were presumably related to abnormally great turnovers of nucleic acids. Time relationships appear to be consistent with this interpretation for  $RNA$  and some fractions of  $DN A$  [269] are known to have turnover times of the order of 2 weeks.

In patients of this category given glycine  $N^{15}$  the concentration of isotope in urinary uric acid was distinctly less in the first few days than in normal man or in patients with primary gout. Yu et al [267] interpreted this as indicating a diversion of glycine from direct to indirect metabolic pathways of incorporation into uric acid. However if this were true the cumulative incorporation of isotope should also be less than normal during the first few days and this was not found. Moreover when glycine  $1-C^{14}$  was given in tracer doses to a patient with polycythemia vera and secondary gout and to another with myeloid metaplasia the initial specific activity values in urinary uric acid were of the same order as those found in control subjects and the cumulative total incorporation of  $C^{14}$  into uric acid was greater in both patients from the outset [213]. In addition the urinary purine bases were very highly labeled on the first day following glycine  $1-C^{14}$  administration in the patient with myeloid metaplasia. Even more striking labeling was observed in urinary purines especially hypoxanthine and xanthine in a matter of hours following administration of AIC  $4-C^{14}$  to a patient with chronic myelogenous leukemia (Fig. 21-17) [214]. These results illustrate the active operation of several shunt pathways involving various nucleotides in these subjects.

individuals predisposed toward development of clinical gout because of inheritance or a blood dyscrasia acute gout is the exception and is sporadic and unpredictable in its occurrence there is no correlation between the uricosuric effect of a drug and its usefulness in acute gout there is no consistent change in the serum or urinary uric acid preceding during or following an acute attack of gout the precipitation of acute attacks by surgery or the rebound attacks following cessation of ACTH or steroid therapy are not easily explained on the basis of effects upon uric acid metabolism *per se* Additional arguments have been presented by Hench [47] and by Gutman and Yu [13]

Since overproduction of uric acid has been demonstrated in both primary and secondary gout it is logical to turn suspicion upon known precursors of uric acid which are common intermediates in both types of exaggerated uric acid production Presumably these would include the precursors of purine nucleotides their interconversion products and their degradation products but not those solely concerned with nucleotide-nucleic acid pathways Since acute gouty arthritis is characterized by florid inflammatory and pronounced local vasomotor changes it is pertinent to consider the propensities of purine compounds to produce such changes Uric acid itself is relatively inert pharmacologically except for effects caused by its precipitation in renal tubules The pharmacologic effects of purine bases ribosides and ribotides have been thoroughly reviewed by Drury [270] Guanine and hypoxanthine together with their respective ribosides and ribotides have little pharmacologic action Xanthine also is relatively inert except for its mild diuretic effect Adenine in small doses has little effect other than mild leukocytosis although in larger doses it is highly toxic to the kidney because of deposition of a highly insoluble oxidation product 2,8-dihydroxyadenine [271] However adenosine and its various phosphorylated derivatives are known to have potent cardiovascular effects when injected in small doses including peripheral vasodilation and local inflammatory reactions These actions of adenylic compounds are impressive enough to have prompted the suggestion that they may play a role in the production of inflammation following injury [270] Despite the variety of effects produced by the substances nothing suggesting acute arthritis has yet been attributed to adenylic compounds except by Thannhauser and Bommes [272] and efforts by Gutman and Yu [13] to confirm these results have been unsuccessful

With the view of exploring the possibility that one immediate cause of acute gouty arthritis whether in primary or secondary gout might be some purine other than uric acid Gutman and associates have studied the excretion of purine bases in urine in a limited number of patients with primary gout during and between acute gouty attacks They [180, 181] reported that excretion of succinadenine is elevated during interval

The addition of a greatly expanded nucleic acid purine pool, or at least of a nucleic acid pool with a greatly accelerated turnover need not therefore introduce any new reactions into the metabolic schemes already discussed. The likelihood that the intermediates employed in overproduction of uric acid in both primary and secondary gout are to a large extent common ones despite the difference in etiology and pathogenesis may eventually permit construction of a unified concept regarding derangements responsible for acute gouty attacks in these two forms of gout.

## ACUTE GOUT

A century ago A. B. Garrod proposed that acute gout was caused by crystallization of urate in tissue and that the inflammatory reaction and paroxysm of gout were consequences of this process.

There is surprisingly little direct evidence for or against this interpretation although there is considerable inferential evidence against it. It is true that urate crystals may be found within the synovium of a joint involved in its first acute gouty attack, but some synovial biopsy samples obtained during an attack have failed to disclose crystals. Conversely urate deposits may be found in synovia of joints which have never been the site of acute gout and large periarthritic accumulations may develop with only indolent reaction on the part of the tissue.

Typical attacks of acute gouty arthritis occur most commonly in a genetically predisposed male with preexisting hyperuricemia. They also occur in hyperuricemic patients with excessive turnover of nucleoprotein purines, i.e. in secondary gout. Recently, acute attacks have also been reported in patients without known predisposition from either of these factors during treatment with pyrazinamide [269] or chlorothiazide [226], two agents which produce hyperuricemia through effects upon renal excretion of uric acid. The common denominator of acute gouty arthritis would therefore seem to be hyperuricemia, but this too appears to have certain exceptions. Many qualified observers have encountered occasional patients in whom unquestionable acute attacks have occurred in the presence of normal serum uric acid levels as determined by reliable methods. Also there is a tendency for patients with gout to experience more frequent acute attacks shortly after beginning the regular use of probenecid, while the serum uric acid level is low.

Additional observations tending to cast doubt upon the direct or exclusive role of uric acid per se in the acute gouty attack include the following: intravenous infusion of uric acid in doses of 20 to 100 mg per kg in normal [229] or gouty [63] persons will not precipitate an acute attack, nor will oral feeding or even subcutaneous injection of uric acid around joints [15]. Acute gouty arthritis occurs rarely, if at all, in chronic nephritis with hyperuricemia and nitrogen retention in hyperuricemic

Levin and associates [278-279] made observations during and between 12 attacks in 6 gouty patients and conducted metabolic balances in 2 subjects for 90 and 161 days respectively during spontaneous acute attacks and during attacks induced by withdrawal of ACTH and testosterone. The results indicated no evidence of pituitary or adrenocortical hypofunction in relation to acute attacks of gout and no significant differences between the responses of normal and gouty patients to administration or withdrawal of ACTH. Neither did these studies support the concept of a 'gout cycle' with respect to electrolyte metabolism [287]. Nevertheless speculation continues that temporary relative adrenocortical deficiency or diminished target responsiveness may be implicated in the pathogenesis of the acute seizures.

#### ALLERGY

Many authors have pointed out that certain aspects of the acute gouty attack resemble those of an allergic reaction. These are reviewed in detail by Cutman and Yu [18] and by Bauer and Calkins [30] and the inadequacies of the hypersensitivity theory are emphasized. Recently Villa Robecchi and Ballabio [164] have again suggested allergy as an explanation of the inflammatory reaction of acute gout while attributing the hyperuricemia and tissue accumulations of uric acid to concomitant increases of production and of tubular reabsorption of urate.

### METABOLIC ASPECTS OF THE TREATMENT OF GOUT

The objectives of treatment differ according to the stage of the disease. From the standpoint of therapy there are four stages of primary gout: asymptomatic hyperuricemia, acute gouty arthritis, interval gout, and chronic tophaceous gout. In addition special therapeutic considerations exist in secondary gout and in patients who form uric acid stones, whether these are idiopathic or secondary to renal tubular disease or to primary or secondary gout.

#### ACUTE GOUT

It is convenient to consider first the treatment of the acute gouty attack since most commonly it is this event which first brings the patient to the physician. The objective is termination of the attack in the shortest time possible by the mildest means available. Several drugs are useful for this purpose: viz. colchicine, phenylbutazone, corticotropin and cortisone or related steroid products.

##### *Colchicine*

The dramatic effect of colchicine upon the acute gouty attack is not accompanied by any detectable influence upon uric acid metabolism.



phases but declines during acute gout whereas that of 7 methyl-8-hydroxyguanine is normal during interval gout and rises during an attack. They suggested that acute gout is accompanied by an imbalance between adenylyl and guanylyl nucleotides but cautiously refrained from further speculation as to the significance of these observations. Of interest however is the observation that 7 methyl-8-hydroxyguanine is consistently elevated in patients with myeloproliferative disorders predisposing toward development of secondary gout [267]. Further observations on the excretion of purine bases in all phases of primary and secondary gout as well as in patients with unrelated types of inflammation will be of great interest.

Because of the highly specific effect of colchicine in acute gout knowledge of its locus of action may constitute an important clue to the metabolic defect underlying the acute gouty attack. Recently, Laster and Blair [103] have demonstrated that colchicine inhibits uric acid ribose phosphorylase apparently on a competitive basis. Phenylbutazone another drug effective in acute gout, also did so but less effectively while probenecid which is without effect upon acute gout did not inhibit the phosphorylase. These results must be interpreted with caution at this time since the role and indeed the occurrence, of uric acid ribose is not established in man.

#### ROLE OF THE PITUITARY-ADRENAL SYSTEM IN ACUTE GOUT

Since acute gout may be precipitated by such diverse factors as local trauma, surgical procedures, physical overexertion, infection, emotional upsets, and dietary and alcoholic overindulgence, it was a logical development of the unifying concept of stress as propounded by Selye that a role of the pituitary and adrenal alarm mechanism should be proposed in the initiation of the gouty attack. Robinson et al. [273] and Hellman [274] have proposed that acute gout is precipitated in the transitory stage of depressed adrenocortical activity presumed to follow cessation of adrenal activation. The rebound exacerbation frequently following cessation of ACTH or cortical therapy would appear to be interpretable in similar terms. Wolfson et al. [275] have proposed a sluggish reactivation mechanism for restoring promptly to normal the secretion of adrenocorticoid following such temporary administration or stimulation of ACTH. Additionally, Wolfson and Cohn [276] have postulated the secretion of an abnormal adrenal androgen in gout based on low ketosteroid measurements in urine in 11 gouty subjects.

Gutman and Yu [13] have raised several cogent objections to these theories including the finding of distinctly low urinary excretion of ketosteroids in only 1 of 10 patients with gout. A variety of other studies has also indicated that urinary ketosteroids are normal in the majority of patients with primary gout [277].

### *Uricosuric Agents*

The advent of clinically useful and well tolerated uricosuric agents has revolutionized the management of gout and the outlook for the gouty patient. Chronic tophaceous gout should soon be virtually an unknown disease. In the vast majority of gouty patients if the disease is recognized early and managed intelligently disabling tophaceous gout is preventable.

The most useful of the uricosuric agents have been probenecid [281-283] and salicylates [284], corticosteroids [192, 285-287] and phenylbutazone [288]. Of great promise are two recent additions, sulfinpyrazone [289] (a phenylbutazone metabolite) and zoxazolamine [290] which are considerably more potent than prior members of this class of agents.

The molecular basis for the action of uricosuric agents is unknown and indeed can scarcely be known until the processes involved in uric acid absorption or secretion in the renal tubule are understood. Small doses of uricosuric agents cause a reduction of uric acid clearance whereas large doses cause pronounced increases in uric acid clearance. This biphasic effect has been attributed to successive inhibition of secretory and reabsorptive processes respectively [220, 222a]. Striking reductions of the miscible pool of uric acid have been demonstrated after administration of adequate doses of salicylates [190], probenecid [291, 292], phenylbutazone [180, 288] and cortisone [192]. These are probably accounted for entirely by the increment in urate excretion. At least in the case of phenylbutazone an increase in uricoly is or of excretion by nonrenal pathways has been excluded. No significant increase in synthesis of uric acid was demonstrable when phenylbutazone was given for a few days [180] but in the case of one of two normal subjects given this drug for a longer time and in two hyperuricemic patients given probenecid [291] the increment in uric acid excretion exceeded the measured decrement in the miscible pool of uric acid. This suggests either mobilization of uric acid not measured within the pool or a concomitant or subsequent increase in rate of synthesis. In the case of corticotropin there is good evidence for increased uric acid production [192, 280, 287] in addition to the urico-uric effect. The possible direct or indirect effects of urico-uric agents on purine synthesis *de novo* and uric acid production deserve further attention.

The clinical effectiveness of urico-uric agents in preventing progression of tophi and indeed in mobilizing uric acid deposits is now well known. Striking examples of reduction in size of visible tophi and of regeneration and recalcification of bone matrix have been published [251-254]. The shift of uric acid balance in tissue from positive to negative occurs as a consequence of lowering of serum uric acid levels to values sufficiently undersaturated to permit dissolution of tophi. In addition the frequency

This drug does not alter serum concentration or renal excretion of uric acid [39] nor does it have any significant effect upon the mobile pool of uric acid or the rate of its turnover [190-193]. A clue to its possible mode of action is provided by its inhibition of uric acid riboside phosphorylase [153] but since the function of this enzyme and the existence of its substrate uric acid riboside are not established in man the mechanism of action of colchicine in gout remains an enigma. Nevertheless its specificity of action in acute gout provides an important diagnostic test. The disadvantage of colchicine is its propensity to cause violent gastrointestinal upsets when given in doses sufficient to terminate an acute gouty attack. Colchicine itself can be given intravenously with good therapeutic effects and less gastrointestinal toxicity [280] and colchicine by oral or intravenous route is considered the drug of choice for acute gout by many physicians.

#### *Phenylbutazone*

This drug is a potent analgesic agent with remarkable effectiveness in the acute gouty attack [29]. It also has potent actions upon renal transport of uric acid. When given in adequate dosage phenylbutazone appears to be as effective as colchicine in acute gout and when used for only a few days for occasional attacks is quite safe. Its advantage over colchicine is that it causes relief of symptoms without producing violent nausea, vomiting or diarrhea. Phenylbutazone is also an inhibitor of uric acid riboside phosphorylase.

#### *Corticotropin and Cortical Steroids*

ACTH and cortisone or newer derivatives thereof are effective in acute gout although not so dependably as colchicine or phenylbutazone [29]. The mechanism of action is unknown. The tendency for a rebound attack to occur upon cessation of therapy has limited enthusiasm for these agents.

### CHRONIC TOPHACEOUS GOUT

In chronic tophaceous gout one employs measures to prevent further accumulations of uric acid and to mobilize existing deposits as well as measures to prevent further acute gouty attacks. One might add as objectives the prevention or reversal of accelerated atherosclerosis or of parenchymal renal disease but at present the influence of therapy upon these processes has not been adequately assessed. In interval gout the therapeutic goals and measures employed are similar but frequently less extensive. Programs are adequate for satisfactory management.

The treatment of tophaceous gout consists principally of the use of uricosuric drugs, regulation of diet and maintenance colchicine although opinion is divided as to the necessity for and virtues of the last two measures in certain patients.

disease and the patient may experience in their place recurring exacerbations of less acute and less paroxysmal joint pain. These symptoms too may be lessened by the regular use of colchicine.

In a series of 31 gouty patients reported by Gutman and Yu [29] daily ingestion of colchicine for 18 months or more resulted in conspicuous reduction in frequency of attacks in 18 and in 13 instances the response has made the difference between virtual incapacitation because of frequent interruption of activities and restoration of full employment. Talbott's [294] experience confirms that presented above.

#### INTERVAL GOUT

The management of interval gout embodies the principles discussed above except that it is frequently not necessary to institute other than general common sense measures in patients early in the course of the disease. For example, in a gouty patient just recovered from the initial attack with only a moderately elevated serum uric acid level and no evidence of tophaceous involvement one is justified in withholding further colchicine or in omitting uricosuric agents until more specific indications arise since the interval between the initial and the second attack may be years and no drug is completely nontoxic.

#### ASYMPTOMATIC HYPERURICEMIA

The incidence of overt gout in hyperuricemic subjects is not known with certainty but may be of the order of 10 to 20 per cent. In the remaining group an occasional patient may develop parenchymal renal disease and some will develop renal stones. Since the majority of asymptomatic hyperuricemic persons appears to suffer no ill effects from this abnormality no treatment is generally indicated. A high fluid intake, control of weight, and avoidance of high purine foods would seem only sensible. The early recognition of a hyperuricemic male in a family in which severe disabling gout is frequent, especially if the gout occurs at an early age, would constitute an indication for more extensive prophylactic measure.

#### SECONDARY GOUT

The treatment of secondary gout is based upon the same principles applied in the management of primary gout with one important potential exception. These patients frequently have basal uric acid excretions of considerable magnitude and the administration of a uricosuric agent must therefore be undertaken with caution if at all, lest renal stone formation or tubular or ureteral blockade with uric acid occur. This is an ever present hazard and also occurs all too frequently following treatment of the primary disease with cancer chemotherapeutic agents, radiation [16] or steroids [169].

of attacks of acute gout may eventually be considerably reduced in patients receiving uricosuric agents, although shortly after institution of therapy acute attacks may be frequent [283]

Any patient with primary gout who shows clinical or roentgenographic evidence of tophaceous involvement should be treated with an appropriate uricosuric agent. In patients with severe renal disease in whom the filtered load of uric acid presented to the tubules is small, use of a potent agent is necessary in order to secure a useful uricosuric effect.

### *Diet*

Since potent uricosuric agents have become available the necessity for rigid dietary control of purine intake is seldom encountered. The major objectives of dietary regulation have therefore become those of common sense such as weight reduction in the large number of gouty patients who are obese and abstinence from high purine foods so as to avoid unnecessary addition of exogenous purine to the uric acid pool. In addition, a moderate limitation of protein intake may be advisable since synthesis of uric acid *de novo* is augmented in both normal and gouty subjects by protein loading [129]. Patients with moderately restricted consumption of protein and purine show an average decline of serum uric acid level of 1 to 2 mg per 100 ml. High fat feedings may influence adversely renal excretion of uric acid [293] through unknown mechanisms. Methylxanthines such as caffeine and theophylline are converted in the body to methyluric acids which play no role in gout. Therefore coffee, tea and other foods or drugs containing methylxanthines need not be restricted. Many patients tolerate alcoholic beverages well. Others find beer or wine much more likely to lead to gouty attacks than distilled beverages. These are highly individual responses.

If the combined use of the general dietary regulations discussed above and a uricosuric agent maintain the serum uric acid level below 5 mg per 100 ml little will be gained by more spartan dietary prescriptions. In patients in whom even large doses of uricosuric agents are not adequate to achieve this goal or in whom tophaceous involvement is severe or rapidly progressive a rigid low purine diet may be indicated. Diets containing about 30 mg purine  $\backslash$  per day are palatable and well tolerated but diets below this figure require omission of most meat and many vegetable items.

### *Maintenance Colchicine*

The daily administration of small doses of colchicine (0.5 to 2.0 mg) is frequently effective in reducing appreciably the number of acute attacks experienced by the patient with gout. In the stage of advanced tophaceous gout acute attacks may be less common than earlier in the course of the

the publication of the historic experiment of A B Garrod in 1848. The first recorded observation of asymptomatic hyperuricemia in a man in whose family gout occurred was made by Folin and Lyon in 1913 [301]. In 1931 A E Garrod [302] classified gout as an inborn error of purine metabolism and suggested that gout would probably prove to be due to a dominant Mendelian factor which is expressed fairly commonly as hyperuricemia but only infrequently as gouty arthritis.

The occurrence of asymptomatic hyperuricemia among family members of gouty patients was emphasized by Jacobson [11] who examined serums from 1 male relative of each of 3 gouty patients comprising 3 sons and 1

TABLE 21-6 HYPERURICEMIA IN RELATIVES OF GOUTY PATIENTS

| No. of gouty patients | No. of relatives | % of gouty relatives | % of hyperuricemic relatives | Percent hyperuricemia | Reference |
|-----------------------|------------------|----------------------|------------------------------|-----------------------|-----------|
| 27                    | 136              | 3                    | 34                           | 20                    | [303]     |
| 13                    | 87               | 3                    | 21                           | 24                    | [304]     |
| 44                    | 136              | 0                    | 16                           | 11                    | [10]      |
| 3                     | 20               | 11                   | 21                           | 20                    |           |
| 3                     | 261              | 16                   | 71                           | 22                    | [15]      |

Wilson D, Collins D H, and Mason R M. *Cout D*. *us n* *l* *ro* *oy* *Soc Med* 44: 285 1951.

SOURCE: C J Smyth [20].

brother and found hyperuricemia in all 3 persons. The first extensive study was that of Talbott [303] who reported in 1940 that 20 per cent of 136 asymptomatic blood relatives of 27 gouty patients had hyperuricemia. In 10 of 27 families at least 1 person in addition to the index patient had a serum urate value in excess of 6 mg per 100 ml.

A number of more recent studies of similar nature has also appeared. These are summarized in Table 21-6. The conclusion appears well established that hyperuricemia is a frequent finding among relatives of gouty patients.

Smyth, Cotterman, and Freyberg [304] have plotted their data on serum urate levels in gouty patients and relatives in a frequency diagram. All their gouty patients had urate levels of 6 mg per 100 ml or greater. The distribution of values among relatives was bimodal for both males and female, the nadirs in these distributions being 6.0 and 5.0 mg per 100 ml respectively. Using these critical levels they classified 10 of 48 male relatives and 11 of 39 females as hyperuricemic. The distribution of hyperuricemic individuals in 19 pedigrees conformed rather closely with expectations if such kindreds were segregated for a dominant autosomal gene for hyperuricemia. If this is the mode of inheritance, the ratio of hyperuricemic to normal offspring of matings in which only

## RENAL STONES

In patients given urico uric agents alkali and large fluid intake should also be administered. The alkali will increase the solubility of uric acid in urine and may counteract the natural tendency of patients who excrete a highly acid urine [29] to form uric acid stones. The bedtime administration of chlorothiazide or acetazolamide may be a useful adjunct in maintaining an alkaline urine.

## SUPPRESSION OF URIC ACID SYNTHESIS

Recently azaserine [137] and diazo-oxy norleucine [138] have been shown to reduce the incorporation of labeled glycine into urinary uric acid, presumably by inhibition of purine synthesis *de novo*. At present these substances are too toxic and their efforts too difficult to control to provide a useful therapeutic avenue but an attractive goal of the future remains that of pharmacologic regulation of synthesis of uric acid *de novo* in the gouty individual.

## DESTRUCTION OF URIC ACID

Recently the infusion of highly purified uricase has been shown to bring about a transient reduction in serum uric acid level in two gouty subjects [296]. This demonstration is of theoretic interest but does not constitute a practical form of therapy at present.

## GENETIC CONSIDERATIONS

Gout has been recognized as a familial disorder from the dawn of medical history. The first recorded expression of this belief is that of Galen (A.D. 131-201) who ascribed gout to debauchery, intemperance and an hereditary trait. Among ancient and more recent writers Cadogan (1675-1726) stood alone among the many who have written on this topic in believing that gout is not hereditary [9].

Modern studies have recorded a familial incidence of gout ranging from 6 to 81 per cent. English observers [297] have reported the familial incidence of overt gout as 38 to 81 per cent in their cases whereas American observers have generally reported lower figures ranging from 6 to 18 per cent in various series [293-299]. The reasons for these differences are not entirely clear although the heterogeneity of the American population may make it more difficult to obtain accurate familial data here than in England. That these reports need not reflect a true difference in inheritance in the two countries is shown by the data of Talbott of Buffalo who has observed a familial incidence in 75 per cent of his cases [300].

The existence of hyperuricemia in overt gout has been known since

with the enzymatic spectrophotometric method of Kalkar and Praetorius. They examined 261 siblings of 32 patients with gout and discovered 71 hyperuricemic individuals as well as 16 definite and 2 doubtful cases of gout among these relatives. Plots of serum uric acid values in both controls and relatives of gouty patients described normal distribution curves. No clear separation could be made between normal and abnormal uric acid values although the mean values for both male and female relatives of gouty patients were higher than the mean values of their respective control series. An attempt was made to fit data on relatives to the sum of two normal distribution curves, a fit which should exist if inheritance were of an autosomal dominant pattern. The data on male siblings conformed satisfactorily but that on female relatives did not unless it were assumed that the heterozygous state were ten times as common as the homozygous normal state in women, an assumption without warrant. They therefore concluded that dominance with incomplete penetrance and recessive heredity were excluded and suggested that cumulative gene action (polymerism) might be operative. This would mean that the level of uric acid in blood was determined by the cumulative contribution of several perhaps many genes and that hyperuricemic individuals represented the extremes of this trait. In this respect hyperuricemia might be compared with tallness, a trait for which definition is arbitrary but which is found more frequently in males and is more pronounced in degree in those with a close relationship to tall males. The hereditary components in stature are best explained on the basis of an interplay between a large number of genetic factors.

The studies of Smyth and Stecher and associates have dealt with different generations within gouty families whereas those of Hauge and Harvald have dealt with siblings of gouty patients. Whatever the precise genetic mechanism or mechanisms underlying the gouty defect it is clear that the trait for hyperuricemia behaves as a dominant factor in most gouty families. An interesting pedigree of a family probably homozygous for the hyperuricemic trait has been published [13].

It is of interest that the three genetic studies discussed above will permit the interpretation of monomerism and autosomal dominance when the analyses are restricted to male, and that Hauge and Harvald have discarded this theory solely on the basis of poor fit of data obtained on female. If indeed penetrance is low in women and expressivity hindered it may be very difficult to choose between dominance with failing expression and polymerism. Such situations defy precise genetic characterization at present.

An estimate of the relative frequency of hyperuricemia and gout may be made from existing data. In a series reviewed by Smyth [20] there were 33 definite cases of gout among 163 hyperuricemic relatives representing predominantly American and Danish families. One may take



one parent is hyperuricemic should be 1:1. There were no hyperuricemic sons among 14 below the age of 16 years. However, when sons above 16 years of age were considered the ratio was 6 hyperuricemic to 7 normal sons. Thus the data concerning male relatives were in agreement with the hypothesis of dominant autosomal inheritance if one assumed that the metabolic change resulting in hyperuricemia is one which is not manifested in males until about the age of puberty. Among daughters of the same matings 4 were hyperuricemic, 10 normal. Two of 6 below 16 years of age and 3 of 4 above 16 years were abnormal. These data on females cannot be used as evidence for an autosomal dominant pattern of inheritance. However, data in females are less easily interpreted since hyperuricemia frequently does not develop even in genetically endowed individuals until after the menopause.

Stecher, Hersh, and Solomon [12] have published data which permit similar analyses. A frequency distribution plot of uric acid values of 137 relatives of gouty persons (excluding spouses) was shifted toward high values in comparison with a similar plot of 1,024 determinations on individuals from the general hospital population. It was also suggestively bimodal, the nadir being 6.0 to 6.4 mg per 100 ml, and 23 of 147 determinations on 137 relatives fell above this range.

Among relatives they observed hyperuricemia in 15 to 21 per cent of mothers, brothers, sisters, and sons, but in none of 45 daughters of index cases. These workers tentatively concluded that hyperuricemia was an autosomal dominant trait with low penetrance in both sexes, but considerably lower in females than in males. When this conclusion was put to numerical test for male offspring only, a correction being applied for small family size, the expected number of hyperuricemic individuals was 31. Actually 26 were found among 51 men. These figures were regarded as showing satisfactory conformity with the expected 1:1 ratio. The data were then used to estimate penetrance. Since 20 hyperuricemic sons were observed where 31 were expected, the penetrance was estimated at about 84 per cent in heterozygote males. And since there were only 8 hyperuricemic females compared with 54 hyperuricemic males, despite nearly equal sex distribution of the 203 individuals of the study, the penetrance was estimated to be about one seventh as high in women as in men, or about 14 per cent. Since not a single daughter of a gouty patient was found to be hyperuricemic among 45 tested, no critical analysis could be applied in the case of female offspring. Both these groups agreed that hyperuricemia could not be attributed to a sex-linked gene, since male transmission was well documented and frequent. Also a recessive genetic transmission pattern was excluded by Stecher et al. on the basis of a probability analysis.

More recently Hauge and Harvald [15] in Denmark have reexamined the problem of inheritance of hyperuricemia. All analyses were performed

creased the affinity of PP ribose P amidotransferase for PP ribose-P or decreased its affinity for ATP or ADP. Either aberration might permit greater than normal synthesis of phosphoribosylamine despite a normal 1 P ribose-P to (ATP + ADP) ratio.

An increase in the availability of glutamine might push phosphoribosylamine synthesis. The effect of high protein diets in augmenting purine synthesis has already been discussed from this viewpoint. If there is a greater than normal availability of glutamine for purine synthesis in gout, it is not reflected in the plasma levels of glutamine [170].

Overincorporation of glycine 1- $C^{14}$  into urinary uric acid has been demonstrated in a sizable percentage of patients with gout. In addition, gouty patients show excessive utilization of AIC for uric acid synthesis, and administered AIC has less of an inhibitory effect upon glycine incorporation into uric acid in gouty than in normal man.

These results have at least one potential common explanation: that of a relative excess of the active ribose compound 1 P ribose-P in the patient with primary gout. A high steady state concentration of PP ribose P would result in generation of additional phosphoribosylamine provided that saturation of the amidotransferase had not already been reached by the existing level of PP ribose P. This would result in the utilization of a larger than normal quantity of glycine. If the various control mechanisms governing interconversion of purine ribonucleotides and synthesis of nucleic acids are competent, prompt conversion to uric acid of surplus purine nucleotides would be the anticipated result. Under these circumstances the activity of 1 P ribose-P amidotransferase would still be viewed as controlling the overall rate of purine synthesis *de novo*, and the rate would be augmented because of elevation of the 1 P ribose-P to (ATP + ADP) ratio.

A high steady state concentration of 1 P ribose-P would also permit conversion of a larger than normal quantity of administered AIC to its ribonucleotide by direct reaction of AIC with this active ribose compound. Prompt conversion of AIC ribonucleotide to purine ribonucleotides followed by cleavage and oxidation of purine bases to uric acid would result in the appearance of a greater percentage of the test dose of AIC in uric acid in gouty than in normal man. In addition, under these circumstances a given quantity of AIC would deplete the supply of 1 P ribose-P less in a gouty than in a normal subject, potentially also explaining the smaller reduction in synthesis of uric acid *de novo* from glycine in the gouty subject when AIC is administered.

An interesting observation potentially constituting a model for study of mechanisms controlling uric acid synthesis *de novo* is provided by studies of Krakoff and Balis [306] who noted that patients given 2-ethylamino-1,4-thiazole promptly showed a remarkable increase in serum and urinary levels of uric acid (while also developing toxic lesions within

<sup>33</sup>/<sub>163</sub> or one fifth, as an estimate of the proportion of hyperuricemic individuals who at any given time will manifest gout. If the estimate [30] is accepted that about 4 per cent of the 12 000 000 arthritic patients in the United States (population, 175 000 000) have gout the incidence of gout would be 274 per 100 000 or 0.27 per cent. The frequency of all persons carrying the genetic determinants of hereditary hyperuricemia would then be of the order of 1.4 per cent or 1 in 71 individuals. The expected incidence, based upon statistical definition of hyperuricemia, would be 2.8 per cent. Talbott [39] found 68 hyperuricemic persons among over 1,500 males tested at the time of routine college medical examinations an incidence of about 1 in 22 individuals (4.5 per cent). These values may not be appropriate estimates in countries such as England and India [30-297] where gout is said to be much more common than in the United States and Scandinavia.

In the study of Hauge and Harvald [15] no linkage was found between predisposition to hyperuricemia and the genes concerned with blood grouping systems ABO, Rhesus MNS P, Lewis Duffy, and Kell.

### HYPOTHESIS REGARDING THE FUNDAMENTAL GOUTY DEFECT

In primary gout considerable evidence now points toward overproduction of uric acid as at least one underlying mechanism responsible for hyperuricemia. According to present concepts sustained overproduction of uric acid can only occur if there is sustained overproduction of purine nucleotides. The key intermediate in the production of purine nucleotides may be phosphoribosylamine for reasons discussed earlier in this chapter.

If phosphoribosylamine does occupy such a critical role then the rate of its synthesis may be an important factor in regulating uric acid synthesis. As a working hypothesis the author would suggest that there is excessive synthesis of phosphoribosylamine in primary gout. The most immediate factors governing the rate of synthesis of phosphoribosylamine will be (1) the activity of its synthesizing enzyme PP ribose-P amidotransferase as determined by the innate characteristics of the enzyme, the quantity of enzyme, the action of inhibiting nucleotides and perhaps other factors; (2) the concentrations of its substrates, namely glutamine and PP ribose-P.

The synthesis of PP ribose-P amidotransferase is undoubtedly under genetic control. The gouty individual might have a greater than normal quantity of the enzyme, perhaps if the magnitude of urate synthesis is a continuous function having a Gaussian distribution in the general population the gouty subject is one very high on the upper limb of the distribution curve. This notion would accord with the genetic data of Hauge and Harvald [13]. Or an enzymatic aberration might have in

occur solely as a consequence of renal<sup>4</sup> retention of uric acid is difficult to harmonize with existing concepts of the disease

## SUMMARY

1 Primary gout is a genetically determined disorder of purine metabolism in which the cardinal feature is hyperuricemia. It is preeminently a disorder of adult males and is characterized both by recurrent attacks of acute arthritis of unknown cause and by a chronic arthritis associated with deposits of urate in joints and other tissues.

2 Hyperuricemia is defined on the basis of statistical analysis of the distribution of serum uric acid values in the general population. In males hyperuricemia is indicated by values above 6.3 mg per 100 ml by colorimetric methods or above 7.5 mg per 100 ml by enzymatic spectrophotometric methods. In females normal values average about 20 per cent lower than in males.

3 Asymptomatic hyperuricemia is approximately five times as frequent as clinical gout in families of gouty persons. Two types of analysis suggest that the incidence of hyperuricemia is 1 to 2 per cent in the general population. It ranged up to 72 per cent in gouty families.

4 Hyperuricemia cannot be attributed to a defect of uricolysis. Evidence suggests that only 2 to 4 per cent of the turnover of uric acid can be attributed to uricolysis in tissues of man, but that perhaps 25 to 30 per cent can be attributed to bacterial breakdown in the gastrointestinal tract. This fraction is possibly increased in hyperuricemic subjects.

5 The renal mechanism for excretion of uric acid has until recently been viewed as one of glomerular filtration followed by tubular reabsorption of all but 5 to 10 per cent of the filtered urates. Renal tubular secretion has now been demonstrated to occur in man, so that interpretation of clearance data in gouty subjects is uncertain. Values for mean net tubular reabsorption of uric acid in gouty subjects are slightly greater than in nongouty subjects at equivalent filtered urate loads. Statistically significant differences in mean values exist between nongouty subjects and asymptomatic hyperuricemic patients, gouty subjects with normal urinary urate excretion and gouty subjects with habitually excessive urinary urate excretion. In the latter group overproduction of uric acid is virtually certain.

6 Overincorporation of glycine or other precursors into uric acid is demonstrable in many cases of gout. Gouty subjects who excrete abnormally large quantities of uric acid have shown overincorporation of labeled glycine into uric acid whenever studied. These patients are representative of about 8 per cent of the gouty population. Gouty subjects who excrete normal quantities of uric acid have shown overincor-

the mouth) The elevation of uric acid production was due to an enhancement of synthesis *de novo* [306-307] rather than to acceleration of catabolism of nucleoprotein purine moieties The incorporation curves of labeled formate and glycine into uric acid resembled those seen in gouty subjects who overexcrete uric acid The oral lesions and the increased levels of uric acid were promptly corrected by administration of large doses of nicotinic acid [306] or nicotinamide [307] and precursor incorporation curves were then again normal Although the mechanisms of these drug actions have not yet been elucidated it is possible that they involve a common point in the pathways of purine and nicotinic acid metabolism viz., PP-ribose-P If thiadiazole blocks endogenous production of nicotinic acid or binding by its ribonucleotide pyrophosphorylase a sparing effect upon PP-ribose-P would result and might lead to an augmented synthesis of phosphoribosylamine and purines at the expense of DPN (a possible explanation of the pellagra-like mouth lesions) Administration of nicotinic acid might now divert PP-ribose-P from purine synthesis toward its normal product, nicotinic acid ribonucleotide This explanation though speculative is provocative from the standpoint of the metabolic defect of gout

An increase in the steady state concentration of PP-ribose-P at the site of PRA synthesis might derive from one of two major groups of defects (1) those capable of resulting in decreased utilization of a given amount of PP-ribose-P in reactions other than that concerned with purine synthesis *de novo* (i.e., generation of phosphoribosylamine) (2) those capable of resulting in excessive production of PP-ribose-P In the latter case gout might be viewed fundamentally as a disorder of pentose metabolism rather than intrinsically as a disturbance of purine metabolism

PP-ribose-P is generated from ribose 5-phosphate and ATP Ribose 5-phosphate has three primary routes of synthesis overactivity of any of which might result in excessive production of ribose 5-phosphate and of PP-ribose-P A possible explanation of the common association of hypercholesteremia and hyperuricemia [308-309] is that both conditions arise from excessive activity of the phosphogluconic acid oxidation pathway increased generation of reduced TPN leading to enhanced cholesterol synthesis and increased generation of ribose 5-phosphate to enhanced synthesis of PP-ribose-P, phosphoribosylamine and purines

These considerations leave open the possibility that genetic effects at different loci might give rise to a common metabolic disturbance manifested as primary gout caution is urged in adopting too readily a unified genetic concept of the disease In this connection it should be recalled once more that gouty subjects do show a low net renal clearance of uric acid Whether this represents a disturbance related in some way to overproduction of uric acid or whether it is an unrelated derangement cannot now be stated although the concept that primary gout might

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poration of glycine  $1\text{ C}^{14}$  in about one half the cases studied. These patients are representative of about 68 per cent of the gouty population. Several factors were cited such as greater dilution within an expanded uric acid pool, or greater gastrointestinal loss in the hyperuricemic subjects which might account for an apparently normal glycine incorporation value despite existing overproduction of uric acid. Certain studies of 4-amino-5-imidazolecarboxamide incorporation into uric acid and of the rate of turnover of the uric acid pool strongly suggest that overproduction of uric acid occurs in gouty subjects more frequently than can be demonstrated at present by glycine incorporation studies.

7 Although studies of renal excretion of uric acid and of glycine incorporation into uric acid in gouty subjects show overlap of results with those of nongouty subjects, the mean values of groups indicate significant abnormalities of both in patients with gout. The question is raised whether the metabolic derangement of gout results in excessive production of uric acid and also of some hypothetical substance which interferes with uric acid excretion by the kidney.

8 Overincorporation of labeled precursors into uric acid in primary gout appears to occur by way of a shunt pathway, or pathways by which a percentage of newly formed purine nucleotides is broken down to purine bases which are then promptly oxidized to uric acid. A hypothesis is presented that overproduction of purine nucleotides occurs because of a defect in regulation of synthesis of phosphoribosylamine, the first specific purine precursor. Mechanisms may exist for regulation of the rates of synthesis of adenylic and guanylic acids so that excessive synthesis of their mutual precursor, inosinic acid results in cleavage of the surplus and direct synthesis of uric acid.

9 The mechanisms underlying the acute gouty attack remain unknown. It is not established that crystallization of urate in the synovium is causally related to the paroxysm. Demonstration of alterations in urinary excretion of certain purine bases during the acute attack, and of inhibition of uric acid riboside phosphorylase by colchicine, have drawn attention to possible metabolic causes of the attacks. Chronic gout is related to accumulations of urate in joints and to associated osteoarthritic changes.

10 The heredity of the hyperuricemia of gout is well established. Whether the hyperuricemia is determined by an autosomal dominant gene with incomplete penetrance (monomerism) or by a cumulative gene action (polymerism) remains unsettled. Indeed if the disorder is heterogeneous, as appears likely, different patterns of inheritance may exist.

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## Chapter 22

### Xanthinuria\*

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James B. Wyngaarden

Xanthinuria is a rare disorder characterized by the excretion of very large amounts of xanthine in urine and a tendency to form xanthine stones. The only well-documented case of xanthinuria is that described in 1954 by Dent and Philpot [1]. Their patient exhibited a striking diminution of uric acid in blood serum and urine. Urinary stones composed of almost pure xanthine were first identified by Marcet in 1817 [2]. Since that time approximately thirty additional cases have been reported [3-13]. Many of these patients had normal blood or serum uric acid levels and therefore were clearly different from the patient of Dent and Philpot.

### CLINICAL FEATURES

#### *Case of Dent and Philpot [1]*

This patient was a 4½-year-old girl with hematuria and urinary frequency. X-ray examination led to a strong suspicion of a nonopaque stone. The urine contained no excess of calcium, cystine, or amino acids. The patient passed a smooth oval stone measuring 1.5 × 1.0 × 0.5 cm and weighing 0.9 gm. It was translucent to x-rays, contained only traces of calcium and magnesium, and was almost ash-free. In the murexide test it gave a reddish-brown color quite unlike that of uric acid and rather unlike that given by pure xanthine. A hydrolyzed sample yielded traces of amino acids, suggesting that protein was present to the extent of about 5 per cent of the weight of the stone. Scrapings of the stone were dissolved and analyzed by paper chromatography in the presence of various purine markers. The material from the stone matched exactly pure xanthine. The urine was then examined by paper chromatography.

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and shown to contain large quantities of xanthine and no detectable uric acid

Xanthine excretion by the colorimetric method of Williams [14] was about 176 mg per day, or 607 mg per gm creatinine. These values suggested that xanthine, not uric acid, was the end product of purine metabolism in this patient (see Figs 21 8 and 22 2). However uric acid may have been present in small amounts for when the urine was analyzed by the method of Folin and Trimble [16] it appeared to contain 30 mg uric acid per day.

The plasma was also examined chromatographically. It showed neither uric acid nor xanthine even when five times the amount required to show uric acid in normal plasma was employed. These results indicated that if either uric acid or xanthine was present the level was less than 1 mg per 100 ml. Since normal plasma may contain less than 0.1 mg xanthine per 100 ml, a considerable elevation of this compound might have escaped detection by this method (see below). Standard chemical analysis by the method of Folin and Trimble showed 0.5 mg per 100 ml apparent uric acid.

This patient was restudied at age 9 by Dickinson and Smellie [15a]. She had had no further calculi in the interim but clubbing of the calices of the left kidney and enlargement of the right kidney had been detected and her blood pressure had varied between 120/65 and 140/80. By specific enzymatic methods plasma oxypurines were 0.74 mg per 100 ml and plasma uric acid was <0.2 mg per 100 ml. The renal clearance of oxypurines was 94 ml per min per 1.73 m<sup>2</sup> body surface area, a value equivalent to 82 per cent of the simultaneous endogenous creatinine clearance. Fresh urine and plasma contained small quantities of hypoxanthine equivalent to 10 to 20 per cent of xanthine. Stored specimens showed larger quantities of hypoxanthine and also showed more uric acid than was found in fresh specimens.

#### *Other Cases of Xanthine Stones*

Xanthine stones have been found in patients ranging from 4 to 72 years of age. Three quarters of the 31 reported subjects have been males. Two thirds of the xanthine stones have been 'pure' and one third 'mixed'. The mixed stones have frequently contained uric acid or calcium oxalate or phosphate in addition to xanthine.

In no case of xanthine stone other than that of Dent and Philpot has the urinary excretion of xanthine been measured or hypouricemia reported. In the few studied cases all adults the level of blood or serum uric acid has been normal [4, 10, 13] or even somewhat high [9]. In one of three Duke Hospital cases hyperuricemia and increased urinary uric acid excretion were found (1958) in a patient who had had a mixed xanthine-uric acid stone 15 years earlier. Excretion of xanthine and of

other urinary purines [16] was normal. The family also proved to be hyperuricemic. A second adult patient who had also passed a mixed xanthine-uric acid stone had a normal uric acid level. A third subject, a woman who had passed stones since early childhood, had pure xanthine stones at age 28 (1917). No uric acid analyses were performed and she has since been lost to follow up. (Stone analyses were performed by Heywood Taylor by the murexide method. The author is indebted to Dr. Taylor for permission to cite these unpublished cases.)

An interesting case has been reported by Ichikawa [12] in a 44 year old male who had a left nephrectomy performed after a lengthy history of hematuria and negative preliminary study for urinary calculi. The kidney was grossly normal but on palpation was found to be studded with numerous small nodules. The cut surface disclosed many small holes with brownish granular concretions which were round or oval, smooth and friable. Chemical tests revealed these to be pure xanthine and some tubules contained xanthine casts. No other instances of parenchymal xanthine deposits have been recorded.

An observation of potential significance is that certain of the adults who formed xanthine stones were observed to excrete highly acid urine. pH values of 4.0 and 4.9 were recorded in two instances [9, 18]. In the three Duke Hospital cases, urine pH values ranged from 6.0 to 6.5. Xanthine is highly insoluble in acid urine. A defect in urinary acidification such as that described by Henneman and coworkers [17] in some uric acid stone formers might have led to xanthine or mixed xanthine-uric acid stone formation in certain of the reported subjects.

It is clear that a considerable number of the adults who formed xanthine stones did not have the metabolic defect present in the young girl described by Dent and Philpot. The observation that several had normal or even elevated serum uric acid levels probably excludes a block in xanthine oxidation in these subjects. Indeed, it is not established that they had any defect of xanthine metabolism. It may be suspected, however, that xanthine excretion was elevated in at least one subject whose blood uric acid was high. Normal for a 60 year old male patient reported by Taylor and Taylor [10] had xanthine stones weighing 12 gm. If xanthine excretion were normal in this subject (6 mg per day), these stones would represent quantitative precipitation and retention of the cumulative urinary xanthine excretion of 6 years.

Of the 31 known cases of xanthine stones, 6 occurred in subjects under the age of 15. In no case was stone formation recorded as a familial tendency, and in this group no measurements of serum or urinary xanthine or uric acid were recorded except by Dent and Philpot. It is not possible therefore to determine whether any other cases of defect in xanthine metabolism have been observed among xanthine stone formers, although the younger patients and those with large pure xanthine stones would

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mixture is carefully evaporated to dryness. To the yellow residue are added a few drops of 4 N potassium hydroxide. Uric acid forms a purplish violet color at this stage; with xanthine the color is a bright red-orange. Upon further heating the uric acid sample turns yellow and yields a red-orange residue upon evaporation.

The differentiation of xanthine from uric acid is difficult if ammonium hydroxide is used in place of potassium hydroxide as in the conventional murexide test.

**Phosphotungstic Acid Test** Xanthine does not give a color test with phosphotungstic acid where uric acid yields a deep blue color. Xanthine can be carefully oxidized to uric acid with hydrogen peroxide or dichromate [7] and will then give a positive test result.

**Folin's Phenol Reagent** Xanthine and guanine react with Folin's phenol reagent to give a blue color, but adenine and hypoxanthine do not [23]. Uric acid will react but can be destroyed by heating with  $\text{H}_2\text{O}_2$  without affecting xanthine.

**Spectrophotometric Methods** A small amount of material is dissolved in 0.01 N HCl or 0.01 N NaOH with gentle heat and the spectrum of the solution is examined against an appropriate blank. Xanthine exhibits characteristic spectrums as shown in Fig. 22-1 [9]. Pure xanthine stones should permit

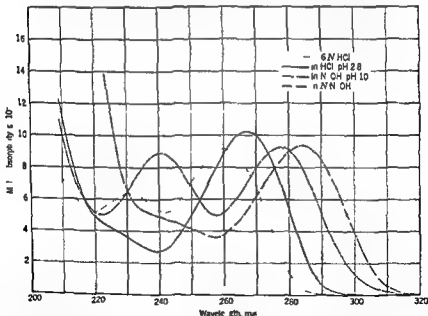


Fig. 22-1 Absorption spectrums of xanthine (I printed from Beaver et al. [9] with permission of the author and the Clirex Inc.)

provisional identification by spectral examination. Upon addition of xanthine oxidase at pH 8.2 the spectrum should change to that of uric acid and by observation at appropriate wavelengths [25-26] the quantity of xanthine present may be estimated. The presence of xanthine in impure stones, particularly those containing uric acid, may be difficult to detect.

constitute the most interesting group in which to search for such a defect.

An early report [18] that serum oxypurine levels are elevated in gout has not been confirmed [19].

### *Other Cases of Hypouricemia*

Hypouricemia occurs in association with a number of inborn errors of metabolism but except in the patient described by Dent and Philpot it appears always to be attributable to failure of renal tubular reabsorption of uric acid. The classic prototype of such a defect is that of the Dalmatian coach hound in which urate and inulin clearances are identical [20]. In man hypouricemia occurs in the de Toni Fanconi syndrome (Chap. 37) and in Wilson's disease (Chap. 25) as a consequence of the renal lesions of these disorders.

An interesting case of hypouricemia has been described by Praetorius and Kirk [21]. They discovered a healthy young man with a plasma uric acid value ranging from 0.2 to 0.6 mg per 100 ml and a urinary output of uric acid of 690 mg per day. Uric acid clearances ranged from 162 to 284 ml per min (normal = 7 to 10 ml per min) and averaged 46 per cent greater than simultaneous inulin clearances. The defect in this subject appears to be that of an extraordinarily high uric acid clearance, possibly including tubular secretion (Chap. 21). The original report also describes an elevated level of "oxypurine" in the plasma of this patient, and in that of his father and son both of whom had normal uric acid levels. Subsequently the high oxypurine levels in these patients were attributed by other authors [22] to breakdown of red cell nucleotides occurring before plasma was separated from erythrocytes. Thus the significance of these elevations of oxypurine levels in this family is unclear.

### *Characteristics of Xanthine Stones*

The majority of xanthine stones have been described as brownish or brown yellow, smooth, round or oval, friable, easily cut with a razor and white and laminated inside. A few have been irregular in shape. They have ranged in size from a few millimeters in diameter to that of a hen's egg [3] and in weight from a few grams to 3 gm [3] or more [10].

Xanthine stones are radiolucent and are generally discerned on pyelography as filling defects only. Occasionally sufficient calcium is entrained within the stone to permit radiographic recognition.

A variety of methods has been used for identification of xanthine in the stone. Most of these leave much to be desired and it is to be hoped that future identification will be made with the highly sensitive and specific methods now available including differential spectrophotometry and paper chromatography.

**Murexide Test.** A small amount of the material is placed in a porcelain evaporating dish, a few drops of concentrated nitric acid are added and the

tion of uric acid (Fig 22 2) <sup>1</sup> The first oxidation step of hypoxanthine is slower than the second one Nevertheless careful studies in vitro have shown that a small but detectable steady state concentration of xanthine does exist during hypoxanthine oxidation [33] This is greater than can be accounted for by intermediate xanthine bound to the enzyme surface and is sufficiently large to constitute a xanthine pool in solution

### XANTHINE OXIDASE

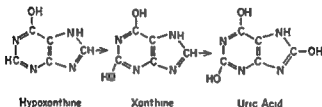


Fig 22 2 Conversion of hypoxanthine to uric acid by xanthine oxidase

Xanthine is formed from guanine by action of guanase. Xanthine must then be acted upon by xanthine oxidase to yield uric acid. Guanase may be found in tissues which lack xanthine oxidase. These include blood [34] and kidney [35] and it is therefore not surprising that urinary xanthine generally appears to have been derived from guanine in isotope labeling studies.

Plasma normally contains 0.1 to 0.3 mg per 100 ml hypoxanthine + xanthine [19, 22] and investigations by enzymatic differential spectrophotometric methods indicate that hypoxanthine is the major component of this pair [36]. Both hypoxanthine and xanthine accumulate in the plasma of shed blood [37] and may be increased 100 fold during standing at room temperature for 48 hr. From 10 to 30 per cent of the increment may be due to xanthine which is presumably derived from catabolism of erythrocyte purine compounds [34]. Urinary excretion of xanthine normally ranges from 2 to 8 g (average 4.1) mg per day [30] and is frequently slightly lower in patients with primary gout [33] or with myeloproliferative disorders [39]. Xanthine + hypoxanthine excretion is not influenced by probenecid in man [40]. This suggests that oxypurine bases are not reabsorbed in the renal tubules by the mechanism responsible for urate reabsorption. The clearance of hypoxanthine + xanthine ranges from 15 to 40 ml per min in man and is therefore three to five times the urate clearance.

pK<sub>a</sub> for hypoxanthine, xanthine and uric acid is 8.8, 7.7 and 7.5 respectively [3]. Therefore at pH 7.4 hypoxanthine and xanthine would be present predominantly in their lactam form whereas uric acid would be present chiefly as the monoalkali salt of a mixed lactam-lactam form.



by these methods although an appreciable increase in absorbance at 292  $m\mu$  upon addition of xanthine oxidase would constitute strong evidence for the presence of xanthine since hypoxanthine is so soluble as to be a most unlikely contaminant of the stone. One should however ascertain by suitable analyses with uricase [25-27] that the increment in absorbance at 292  $m\mu$  does indeed represent generation of uric acid.

**Chromatographic Methods.** A number of paper [28] and column chromatographic systems [16-29] is available for separation and identification of xanthine. Movement of the unknown material in a manner similar to that of known xanthine in several systems would constitute strong evidence for identity of the two substances.

A paper chromatographic method was employed by Dent and Philpot in identifying xanthine in the stone recovered from their patient. It is hoped that semiquantitative determinations of all the urinary purines by paper [16-30] or column chromatographic methods [16-29] will be performed on subjects who form xanthine stones so that possible lesser alteration of excretions of other purine bases may also be evaluated.

## XANTHINE

The origin of xanthine has been discussed in Chap. 21 and its relationship to the broader aspects of purine metabolism is presented there. In mammalian systems xanthine has three known immediate precursors: hypoxanthine, guanine, and xanthosine (or deoxyxanthosine) and it is the only established precursor of uric acid (Fig. 21-8).

Presumably the major precursors of xanthine are hypoxanthine and guanine. Each of these bases is formed from its respective nucleoside by action of purine nucleoside phosphorylase, an enzyme which readily cleaves inosine and guanosine but has only limited activity toward xanthosine. Isotope studies discussed in Chap. 21 have provided evidence for significant early labeling of urinary hypoxanthine and guanine following administration of labeled precursors such as glycine or 4-amino-5-imidazolecarboxamide. These observations indicate that the purine bases are products of cleavage of newly formed nucleotides as well as of older nucleotides derived from catabolism of nucleic acids. In most studies the specific activity of urinary xanthine paralleled that of guanine but in some it followed more closely that of hypoxanthine [29-31]. Thus the predominant precursor of urinary xanthine may be either guanine or hypoxanthine in different subjects. Normally only a very small fraction of the xanthine turnover appears in the urine; the large portion by far serves as a precursor of uric acid.

As pointed out above, xanthosine and deoxyxanthosine are relatively poor substrates for nucleoside phosphorylase and presumably little xanthine originates from these precursors. Xanthine arises from hypoxanthine by action of xanthine oxidase as an intermediate in the forma-

TABLE 22.1 RELATIVE RATES OF OXIDATION OF PURINE DERIVATIVES BY BOVINE MILK AND HUMAN LIVER XANTHINE OXIDASES

| Substrate         | Product                                 | Oxidative pathway† | Relative rate with           |                              |
|-------------------|---|--------------------|------------------------------|------------------------------|
|                   |   |                    | Bovine milk xanthine oxidase | Human liver xanthine oxidase |
| Xanthine          | Uric acid                               |                    | 1.0                          | 1.0                          |
| 1-Methylxanthine  | 1-Methyluric acid                       |                    | 0.45                         | 1.0                          |
| 8-Dioxyxanthine   | Uric acid                               |                    | 1.0                          | 1.1                          |
| Hypoxanthine      | Xanthine → uric acid (8-dioxyxanthine?) | → 6 → 2C9          | 0.7                          | 0.7                          |
| Purine            | Uric acid                               | → 6 → 7C → 6C      | 0.3                          | 0.13                         |
| 2-Oxypurine       | 2,8-Dioxyxanthine                       | → 2,8 → 2,6,8      | 0.16                         | 0.01                         |
| 8-Oxypurine       | 2,8-Dioxyxanthine                       | → 2,8 → 7,6,8      | 0.01                         | 0.00                         |
| 8-Oxypurine       | Uric acid                               |                    | 0.00                         | 0.000                        |
| 2,8-Dioxyxanthine | Uric acid                               |                    | 0.00                         | 0.000                        |

Xanthine served as reference compound

† Indicates order in which the oxidizable compounds were attacked by the enzyme

Source: F. Bergmann et al. [33]

variable rates [52]. The enzyme is strongly inhibited by 6-pteridyl aldehyde [53]. Xanthopterin is oxidized to its 8-hydroxy derivative by xanthine oxidase [20].

#### STUDIES ON THE ACTIVE SITE OF XANTHINE OXIDASE

Xanthine oxidase activity towards purines and aldehydes is inhibited by sulfhydryl poisons and by cyanide. A metal mercaptide group has therefore been postulated as an essential part of the active site of xanthine oxidase [54]. Cyanide inhibition of xanthine oxidase presumably involves formation of a stable ferric-cyanide or molybdo-cyanide complex, but binding studies have clearly shown that such inhibition does not interfere with the ability of the enzyme to bind purines. There is evidence that the isoalloxazine ring of FAD constitutes the purine binding site [33, 54] and possibly also the aldehyde binding site [54]. The competition by boric acid with both hypoxanthine and cyanide for the active site suggests that the metal mercaptide groups and the isoalloxazine ring must be located in close proximity.

Fridovich and Handler [54] have proposed a structure for the active site of xanthine oxidase (Fig. 22.3). The active site contains both FAD moieties, only one of which is oxidizable by oxygen or dyes. The metals at the active site are involved in several bonds, i.e. to an adjacent sulfhydryl and to each of the flavins. Electrons pass from the purine substrates to the first flavin and thence via the metal (ferric?) [11].

## XANTHINE OXIDASE

Xanthine oxidase is one of the most extensively studied enzymes. Its distribution in mammalian and avian tissues varies from species to species. In man xanthine oxidase is found in abundance in liver and small intestinal mucosa [41-42] and possibly in bone marrow [42]. It has also been found in human milk [41] but all other human tissues studied have failed to show xanthine oxidase activity. The  $\pi$  include blood, kidney, spleen, heart and skeletal muscle, lung and pancreas [41-42].

Human liver xanthine oxidase has a substrate specificity identical with that of the bovine milk enzyme [33]. This observation implies that information derived from studies of the latter enzyme are relevant to man and this is fortunate since the great majority of careful studies have been conducted with bovine milk xanthine oxidase.

Xanthine oxidase is a flavoprotein containing molybdenum and iron [43]. Bovine milk xanthine oxidase contains two molecules of flavin adenine dinucleotide, one atom of molybdenum and eight atoms of iron per molecule of enzyme and has a minimal molecular weight of approximately 320,000 [44]. Different FAD/Mo/Fe ratios have been reported for the enzyme obtained from mammalian intestine and liver and avian liver [45] but no observations are available on the human enzyme. The enzyme has a remarkably large number of substrates including (1) purines, (2) aldehydes and (3) pteridines. There is now some doubt that oxidation of DPNH by xanthine oxidase preparations is attributable to xanthine oxidase itself [46].

Purine compounds are oxidized in the 2, 6, or 8 position in the presence of xanthine oxidase. The order in which the oxidizable positions are attacked by xanthine oxidase varies from substrate to substrate. With purine the initial attack is at carbon 6. With hypoxanthine the next attack is directed towards carbon 2. Positions 2 and 8 form a closely related pair and oxidation at one of the  $\epsilon$  carbons leads always to attack at the other partner in the next step [33].

Table 22.1 gives data on relative rates of oxidation of purine derivatives by bovine milk and human liver xanthine oxidases. In addition to the substrates shown, xanthine oxidase attacks adenine [47], 2-hydroxyadenine, 8-hydroxyadenine [48], 2,6-diaminopurine [49], 2-azaadenine, 2-azahypoxanthine [50] and a variety of other substituted purines. It does not attack methylxanthines other than the 1-methyl compound [33]. A variety of purine compounds is adsorbed by the enzyme without being oxidized [51]. The enzyme is competitively inhibited by a number of purine bases [49].

**Other Substrates.** All 31 different aldehydes which have been tested are oxidized to the corresponding acid by xanthine oxidase although at

of an increment in xanthine output. If it is much less than 10 ml per min the calculated plasma level would soon be so high as to be inconsistent with experimental data already cited. Even a total inability to reabsorb xanthine in the renal tubule will therefore not explain excretion of xanthine at a level of 600 mg per gm creatinine.

### *Metabolic Block Mechanism*

According to this hypothesis the xanthinuric patient lacks almost entirely the enzyme xanthine oxidase. Such a lack would no doubt lead to an accumulation of xanthine derived from guanine by action of guanase and possibly from xanthine nucleosides by action of nucleoside phosphorylase. The small amount of uric acid in urine might be attributed to dietary sources to a remaining trace of activity of xanthine oxidase or possibly to an accessory pathway such as cleavage of uric acid riboside [56]. The increase in hypoxanthine and of uric acid in urine on standing possibly represents a dismutation reaction



If xanthine oxidase were lacking one would anticipate an appreciable accumulation of hypoxanthine as well as of xanthine in plasma and urine for there is good evidence that free hypoxanthine is normally an important intermediate in uric acid formation and the major oxypurine base normally present in plasma. Several possible mechanisms may be considered to explain a lack of hypoxanthine accumulation, all quite theoretical. (1) A hypoxanthine oxidase distinct from xanthine oxidase may exist. Such an enzyme has been postulated [57] but never identified. (2) Reutilization of hypoxanthine for nucleoside and nucleotide formation may be so efficient as to keep levels from rising abnormally. (3) Xanthine oxidase may not actually be missing but may be structurally abnormal; it may be able to bind and oxidize hypoxanthine but not able to oxidize xanthine.

The last possibility is suggested by recent evidence that the two-step oxidation of hypoxanthine to uric acid involves release of the intermediate xanthine from the enzyme surface and rebinding of xanthine possibly to different active centers of the first IAD component. Bergmann and Dikstein [58] have suggested that hypoxanthine is bound at N1, N3, and N7 while xanthine is bound at N3, N7, and N9. An inability of the enzyme to bind the purine ring at N9 might render it incapable of acting upon xanthine without interfering with its action upon hypoxanthine. Or alternatively such an enzyme abnormality might permit only very slow oxidation of xanthine with production of only very small amounts of uric acid. If opportunity ever permit examination of liver or of breast milk of a xanthinuric subject for xanthine oxidase activity upon various substrates this possibility would be of great interest to explore.

mercaptide groups which are each single electron acceptors to the second flavin from which they may pass to oxygen or other acceptors. Mercury and cyanide both inhibit by interfering with the ability of the metal mercaptide group to transfer electrons from the first to the second flavin.

This hypothesis would explain the occurrence of purine binding by the cyanide-inhibited enzyme and inhibition by high substrate concentration of purines which would also combine with the isalloxazine ring of the

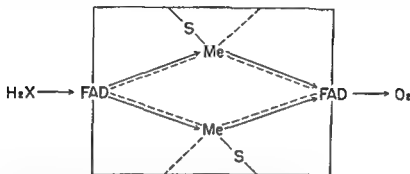


Fig. 22-3 Proposed structure for the active site of xanthine oxidase. (Modified from Fridovich and Handler [54] with permission of the authors.)

autooxidizable flavin and thus prevent interaction with oxygen or other electron acceptors.

## METABOLIC DEFECTS IN XANTHINURIA

Dent and Philpot [1] suggested that gross xanthinuria with very low levels of uric acid in both plasma and urine might be a consequence either of a block in oxidation of xanthine to uric acid or of a 'deviation' mechanism by which xanthine is excreted in urine too rapidly to allow much of it to be oxidized to uric acid. Dickinson and Smellie [15a] have suggested that both defects are present and have published studies showing a renal clearance of oxypurines approaching that of the glomerular filtration rate in the original patient of Dent and Philpot.

### Renal Deviation Mechanism

The deviation mechanism is not adequate as a single defect to explain the known findings. In a normal adult with a glomerular filtration rate of 120 ml per min, an excretion of xanthine of 6 mg per day, and a xanthine clearance of 20 ml per min, the plasma xanthine concentration would be of the order of 0.02 mg per 100 ml. A person with a similar filtration rate and a total inability to reabsorb xanthine would then excrete only 36 mg xanthine per day, and less if the plasma level declined as a consequence of renal xanthine loss. If the normal clearance of xanthine is greater than 20 ml per min, loss of ability to reabsorb xanthine would lead to even less

doubt add greatly to the burden of xanthine excretion and should be limited. Methylxanthines such as caffeine and theophylline are very much more soluble than xanthine and are not metabolized by xanthine oxidase. There would seem to be no unique reason to prohibit their use in xanthinuric subjects.

### SUMMARY

1 Xanthinuria is a rare disorder characterized by the excretion of very large amounts of xanthine in urine and a tendency to form xanthine stones. There is also a striking diminution of uric acid in serum and urine.

2 In only one case that of a girl studied at 4½ and again at 9 years of age have adequate studies been performed to document xanthinuria and hypouricaciduria. Several additional patients have formed xanthine stones in childhood and may provisionally be considered to have xanthinuria.

3 Many of the adults who have formed xanthine stones have clearly not had the defect of the xanthinuric child for in several instances normal or elevated serum uric acid levels were found. Circumstantial evidence suggests that xanthine excretion may have been excessive in a few but this is unproved. In one case xanthine deposits were found in the renal parenchyma.

4 The metabolic defect in xanthinuria probably involves both an absence of (or at least a defect in function of) xanthine oxidase coupled with a renal tubular defect in reabsorption of xanthine.

5 The genetic factors in xanthinuria are wholly obscure except that an autosomal dominant inheritance is precluded.

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### *A Combined Defect*

The studies of Dickinson and Smellie strongly suggest the presence of two defects, namely absence of xanthine oxidase and absence of the renal tubular absorptive mechanism for xanthine. Since human kidney does not contain xanthine oxidase activity [41-42] these authors postulate that a single genetic defect has led to a defect in the synthesis of a specific protein serving as the apoenzyme of two catalysts having different physiologic roles. Alternatively the results raise a question basic to biochemical genetics: does a gene (or genome) control the synthesis of a polypeptide fragment which can serve as a component of two or more larger polypeptide (protein) molecules?

## GENETICS

Thirteen relatives of the xanthinuric subject examined by Dent and Philpot [1] showed no abnormal excretion of xanthine and had normal outputs of uric acid. These included an only sister, both parents, and three surviving grandparents. None gave a history of renal stone, and the parents were not related. No statement regarding genetic factors other than exclusion of an autosomal dominant inheritance is justified at present.

In no instance has more than one case of xanthine stone formation been recorded in a single family. Both sexes have been affected, the sex distribution being 5 males and 1 female under age 15, and about 3:1 in favor of males in adults. Therefore no statement can be made regarding genetic factors involved in xanthine stone formation in general. Indeed the adult cases described may not be dependent upon inherited metabolic factors, since there is presently no compelling evidence that a metabolic derangement exists in many such patients.

## TREATMENT

Prevention of xanthine stone formation in predisposed individual depends upon recognition of the low solubility of xanthine in acid solutions, and of the possible tendency of certain of the affected individuals to excrete a highly acid urine. Dent and Philpot [1] found that 100 ml normal urine at 26°C dissolved 6.7, 6.5, and 16.5 mg xanthine at a pH of 5.8, 7.0, and 8.1, respectively. The  $pK_a$  of xanthine is 7.7, the  $pK_a$  is 10.6 [32].

A high fluid intake and oral alkali in quantities sufficient to maintain an alkaline urine would appear essential. Dietary regulation of purine intake is not indicated in the patient with a normal xanthine output, for xanthine excretion is independent of diet in such patients [16]. However in a patient with a block in xanthine oxidation, dietary purines would no

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literature has not revealed any report of the normal urinary excretion of orotic acid. Examination of the urine of 73 human beings, both normal and with a variety of hematologic diseases, has failed to reveal the presence of the compound, with the single exception of the patient who provides the basis for this chapter [1]. More extensive studies with more sensitive methods may modify the generality of this finding.

### *Biosynthesis of Orotic Acid*

The investigations elucidating the biosynthetic pathways leading to orotic acid and its role in the biosynthesis of pyrimidines have been adequately and recently reviewed [6-8] and all the details need not be repeated here. The salient features of what now appears to be the major pathway of pyrimidine biosynthesis *de novo* are summarized in Fig. 23.1. Several alternative pathways have been shown to exist, but at present these seem to be of relatively minor quantitative importance in mammalian tissues.

The first step shown in Fig. 23.1, the carbamylphosphate synthetase reaction, is not unique to orotic acid biosynthesis; it also participates in the synthesis of citrulline from ornithine. An active intermediate, not yet unequivocally identified, is involved [9]. In bacteria the overall reaction is reversible, but in mammals only the portion after the formation of the active intermediates has been demonstrated to be reversible [10]. The next step, the carbamylphosphate transferase reaction, appears at present to be unique to orotic acid biosynthesis. The equilibrium of this reaction lies so far towards carbamylaspartic acid [11] that it is essentially irreversible. Ring closure in carbamylaspartic acid to yield dihydroorotic acid is effected by the enzyme dihydroorotase, and in this instance the equilibrium lies about 2:1 towards carbamylaspartic acid [12]. The oxidation of dihydroorotic acid is carried out by the DPN-linked dihydroorotic dehydrogenase, with the equilibrium lying far towards the left [13]. It is to be noted, despite these equilibrium points to the left, that the probable presence of systems for the removal of DPNH would favor the accumulation of orotic acid if its further transformations were blocked. This is precisely what happens in some bacteria [14], in *Neurospora* [15], and presumably in the patient who is the subject of this chapter [1].

### *Biosynthesis of Pyrimidine Nucleotides*

Normally, orotic acid is converted to the ribonucleotide, orotidylic acid, by reacting with 5-phosphoribosylpyrophosphate under the influence of the specific orotidylic pyrophosphorylase [16]. This reaction is reversible, with the equilibrium being about 9:1 in the direction of orotic acid. Orotidylic acid is then decarboxylated to uridylic acid, a reaction for which reversibility could not be demonstrated [16]. Uridylic acid may then be converted to other pyrimidine nucleotides, as indicated in the

## Chapter 23

### Oroticaciduria\*

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*Charles M. Huguley, Jr. and James I. Bain*

This chapter describes an abnormality thus far noted only once [1]. It occurred in the child of a consanguineous marriage. It is included in this book because it probably represents a genetically determined disorder of pyrimidine metabolism.

This child developed a severe anemia at age 5 months, characterized by hypochromic erythrocytes and megaloblastic marrow. The disease did not respond to treatment with iron, pyridoxine, vitamin B<sub>12</sub>, folic acid, or ascorbic acid. The most intriguing feature was the passage in the urine of large amounts of crystals which proved to be orotic acid. There was some improvement in all features of the disease after the administration of adrenocorticoids, and almost complete clearing of the evidence of illness after administration of a mixture of uridylic and cytidylic acid.

#### OROTIC ACID

Orotic acid (4-carboxyuracil) was first discovered in cow's milk [2] and has since been isolated from the milk of several species, including man [3]. Even though it is an important intermediate in pyrimidine biosynthesis, orotic acid apparently does not accumulate in any appreciable concentration in animal tissues, since injected C<sup>14</sup>-labeled orotate does not encounter any perceptible metabolic pools in rat liver or blood, and the radioactive orotate disappears as such from the liver within 30 min [4]. The injected radioactive orotate is excreted with unchanged specific activity, and excretion is complete within 2 hr. Using isotope dilution techniques, Smith and Baker [5] were unable to find evidence of orotic acid in plasma, erythrocytes, or leukocytes in normal man. A search of the

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understood as a result of studies such as those from the laboratories of Kornberg [17] Ochoa [18] Kennedy [19] Leloir [20] and many others

The figure shows the central position of orotic acid an obligatory intermediate in pyrimidine synthesis *de novo*. Pyrimidine bases derived from other endogenous sources or from exogenous sources may however be utilized for the formation of more complex molecules such as nucleic acid. For example Cannellakis [21] has shown that uracil may be incorporated into ribonucleic acid at rates comparable to those of orotic acid incorporation when the conditions are such that a favorable competition exists between degradative and anabolic pathways. Uracil and thymine have also been shown to react in liver preparations with deoxyribose 1 phosphate to yield the corresponding nucleosides [22].

Two pathways for the formation of thymidine and its phosphorylated derivatives are known. (1) the better established one proceeds from uracil deoxyribonucleotide (or deoxyribonucleoside) by direct methylation to thymidylic acid (or thymidine). (2) the other proceeds from cytosine deoxyribonucleoside (or deoxyribonucleotide) either by methylation and subsequent deamination to the thymine derivatives or by deamination back through the uracil deoxyribonucleoside pathway. The relative importance of these alternative pathways is yet to be completely evaluated for any mammalian tissue but the second is rendered doubtful by the work of Kit and associates [23].

## MEGALOBLASTOSIS

### *The Roles of Vitamin B<sub>12</sub> and Folic Acid*

The established hematopoietic efficacy of vitamin B<sub>12</sub> and folic acid in various types of megaloblastic anemia makes it appropriate here to consider briefly what is known of their biochemical roles.

Evidence has been advanced indicating that vitamin B<sub>12</sub> may play a role in protein synthesis, deoxyribose synthesis, reduction of sulfhydryl groups, methyl group synthesis, and conversion of folic acid to an active form [24-31]. Nevertheless, despite the obvious importance of this vitamin, its exact roles are not clearly delineated.

The biochemical actions of folic acid while still not completely clear are much better known than are those of vitamin B<sub>12</sub>. Folic acid is primarily involved in the transformation or transfer of one-carbon units in a variety of metabolic processes concerned with purines, pyrimidines, amino acids, and probably other metabolites as well [24-26]. It seems appropriate to consider here only its direct connection with pyrimidine biosynthesis which occurs at the conversion of deoxyuridylic acid to thymidylic acid. The mechanism of this reaction has been postulated as a hydroxymethylation or formylation of deoxyuridylic acid through the intervention of the appropriate form of tetrahydrofolic acid (either

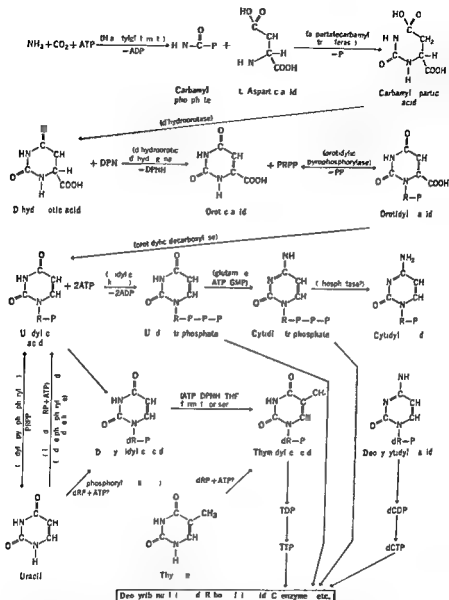


Fig. 23.1 Biosynthesis of orotic acid and pyrimidine nucleotides

figure Much remains to be learned regarding these pathways implicit reliance cannot be placed in the generalizations of the figure

Figure 23.1 carries the pyrimidine transformations only as far as the nucleotide level. The ultimate incorporation of the pyrimidine nucleotides into deoxyribonucleic acid, ribonucleic acid, the corresponding nucleoproteins, and various coenzymes occurs by processes just beginning to be

cytoplasm and nucleus of the cell. The resulting cell is gigantic and the distribution of DNA in the nucleus is sparse and delicate. The cell matures with infrequent mitoses and the basophilic RNA remains in high concentration in the cytoplasm.

Other information of particular relevance is the effect of administration of various pyrimidines and purines to patients with megaloblastic anemia. The purines have been ineffective [39]. Orotic acid [42], uracil [43], thymine [44], thymidine [45-49] and a mixture of uridylic and cytidylic acids [42] have all been effective in some cases though not in all. The effects may be incomplete and are usually short lived. In patients who have responded the dose of orotic acid has been 3 to 6 gm per day, that of uracil 15 to 30 gm daily and that of thymine 10 to 15 gm daily. These ratios are in keeping with what might be inferred from the previous discussion of pyrimidine biosynthesis. Of particular interest are two patients [48, 50, 51] with megaloblastic anemia requiring folic acid who responded to 15 gm daily of thymine after failing to respond to 30 gm daily of uracil. This seems to fit with the demonstrated need for folic acid in the methylation of uridylic acid to thymidylic acid.

The previous discussion presents a basis for understanding the effects of these metabolites in folic acid deficiency but because the exact site or sites of vitamin B<sub>12</sub> activity is unknown there is no explanation for the effectiveness of these metabolites in vitamin B<sub>12</sub> deficiency. One would hardly expect that any of these materials given singly would afford a universal or complete relief of the metabolic defects resulting from deficiency of either vitamin B<sub>12</sub> or folic acid. Indeed it is remarkable how much some patients have improved and how long they have been maintained in remission while receiving only one of these metabolites.

It is not to be assumed that the derangement in nucleic acid metabolism leading to formation of a megaloblastic marrow results only from absolute or relative deficiency of folic acid or vitamin B<sub>12</sub>. It must be remembered that at each step in the metabolic chain an enzyme is necessary as well as the coenzyme (the vitamin). A genetic lack of an enzyme at any point in this metabolic chain (Fig. 23.1) would be expected to produce a megaloblastic anemia. It is only recently that the relative amount of such enzymes present in normal human cells has been investigated [5]. Enzyme deficiency could explain several apparently inexplicable cases of megaloblastic anemia which have been reported including the one detailed below.

## CLINICAL FEATURES OF OROTICACIDURIA

Full details of the clinical course of the only case of this abnormality so far reported have been given elsewhere [1]. This child had a normal delivery at full term and no abnormalities were discovered at birth. His health and development seemed normal until the age of 3 months. From

hydroxymethyl or formyl) accompanied or followed by reduction to a methyl group with perhaps either DPNH or tetrahydrofolic acid acting as the reducing agent [32-34]. In considering the over all etiology of megaloblastic anemia it must be realized that folic acid like vitamin B<sub>12</sub> has manifold points of action in the metabolic sequences of the cell. It is not yet possible to say whether or not there is one point which is more critical than others for the normal development of the erythrocyte and other rapidly proliferating cells.

### *Significance of Megaloblastosis*

The outstanding hematologic abnormality in the condition under discussion is the presence of megaloblasts in the marrow. This morphologic abnormality was originally described by Ehrlich [35] in the erythrocyte precursors. Strictly speaking the term *megaloblast* refers to an abnormal nucleated red blood cell. Similar changes however are observed in the granulocytes and megakaryocytes of the marrow in pernicious anemia [36] and in the epithelial cells of the gastrointestinal tract in the same disease [37].

The abnormality is a gigantism of the cell. The nucleus is large and frequently irregular and has a delicate chromatin pattern and large nucleoli. Basophilia of the cytoplasm persists very late as the cell matures. Reisner [38] has written a critical review of the nature and significance of megaloblastic blood formation.

The occurrence of a megaloblastic marrow is usually due to a deficiency of vitamin B<sub>12</sub> or folic acid and in nearly all instances will respond to the administration of one or both of the vitamins. Deficiency may result from dietary lack or from one of several conditions interfering with absorption. Megaloblastosis may also be seen occasionally in circumstances in which the mechanism is not fully understood but is believed to be due in part at least to increased requirements of one or the other of these vitamins [38]. It can also be produced in man by the administration of anti-folic acid or antipurine drugs and in susceptible individuals by the administration of diphenylhydantoin or primidone.

A few cases of megaloblastic anemia have responded to neither vitamin [39-40]. Crystalluria was not described in these cases.

A second approach to the interpretation of megaloblastic changes is that of Thorell [41] who studied the DNA and RNA content of individual megaloblasts at different stages of development. Reisner [38] has reviewed more recent developments. The quantity of DNA in the nucleus is normal but large amounts of RNA persist in the cytoplasm to a very late stage in the development of the cell. It is theorized that the inability of the cell to synthesize DNA rapidly interferes with mitosis. The cell continues to grow and although the rate of production of RNA may also be subnormal large amounts of RNA are gradually deposited in the

At that time or subsequently a variety of diagnostic studies was made. These studies as well as the patient's failure to respond to specific therapy, eliminated a deficiency of vitamin B<sub>12</sub>, folic acid, pyridoxine or iron as the cause of his anemia. Hemoglobinopathy Mediterranean syndrome and De Guglielmo's syndrome (erythremic myelosis) were also excluded.

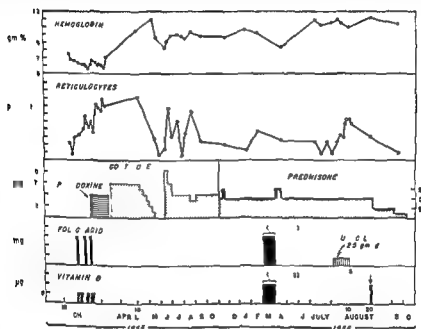


Fig. 23-3 The lack of effect of parenteral folic acid, vitamin B<sub>12</sub>, and pyridoxine and the partial beneficial effect of corticoids on hemoglobin and reticulocytes in a patient with orotic aciduria.

The course of the disease is detailed in Figs. 23-3 and 23-4. Figure 23-3 shows the failure of response to parenteral folic acid, vitamin B<sub>12</sub>, and pyridoxine. Cortisone induced a partial hematologic remission which was later maintained by prednisone or prednisolone. The marrow abnormalities were affected little if at all.

Throughout the course orotic acid was present in the urine whenever it was examined. If a good fluid intake and urine output were maintained, the freely voided urine would contain no crystals, but large amounts would precipitate on standing. When the intake of fluids was reduced, as always happened when the patient felt ill, voiding would be infrequent and crystals would precipitate in the bladder. On several occasions urethral obstruction developed and on one occasion right ureteral obstruction. Fortunately, the obstructions always cleared when fluid



then until the time of his death at 2 years and 9 months of age he was chronically ill with anemia repeated respiratory infections recurrent diarrhea and crystalluria. Prior to hematologic study he had been placed on a low fat diet which controlled the diarrhea and was given medications which he continued to receive for the rest of his life. These assured a satisfactory intake of iron ascorbic acid folic acid vitamin B<sub>12</sub> liver concentrate and other vitamins.

All ancestors were English or Scottish as far as is known except for one (Fig. 232), a founding, believed to be French in origin who was a

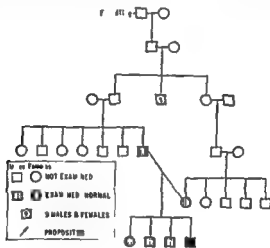


Fig. 232. Pedigree of patient with orotic aciduria.

common ancestor of both parents the great grandfather of the father and the great great-grandfather of the mother. Neither parent nor any of three siblings had anemia abnormality of erythrocytes or orotic aciduria.

When first studied at age 9 months the patient was pale and weak but well developed and apparently well nourished. The clerae were definitely blue. There was torsion of the left tibia outward but no other skeletal abnormality. The tongue had a normal coat. The edge of the liver and the tip of the spleen were just palpable.

A severe anemia was found. The erythrocytes were hypochromic with a striking anisocytosis and poikilocytosis. There were a few nucleated erythrocytes. The reticulocyte percentage was slightly elevated. There was a leukopenia with a monocytosis. The platelet count was normal. The marrow was hypercellular and had striking abnormalities of the megaloblastic type in cells of both the granulocytic and rubricytic series. The urine was negative except for a moderate number of crystals which were later proved to be orotic acid.

Prednisone was discontinued and the patient was permitted to relapse. Figure 23-4 indicates the subsequent response to prednisolone: a moderate reticulocyte rise and improvement in the hemoglobin level, a rise in the white blood cell count and in the percentage of neutrophils. There was a fall in the orotic acid excretion, but the level remained high.

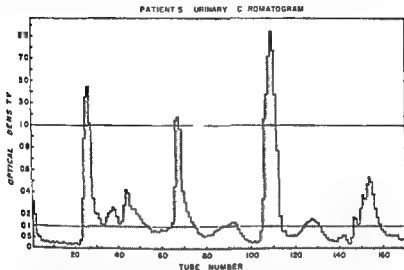


Fig. 23-5. Column chromatogram of patient's urine. Chromatograms of this type were obtained by applying a suitably diluted sample of urine adjusted to pH 8.0 containing approximately 150 to 200 optical density units measured at  $260\text{ m}\mu$  to a  $1 \times 10\text{ cm}$  Dowex 1-chloride column. The column was washed with twice the sample volume of water and eluted with HCl using a continuous gradient elution system [5\*]. The 760-ml mixing flask contained water at the beginning, 435 ml 0.015 N HCl, 200 ml 0.1 N HCl and 100 ml 5% HCl were run successively through the mixing flask from a reservoir under enough air pressure to give a rate of 0.5 to 0.7 ml per min. Then 100 ml 5% HCl was run directly onto the column to remove any uneluted material. Optical density of each fraction was read on a Beckman DU spectrophotometer at 260 and  $285\text{ m}\mu$  but only the  $260\text{ m}\mu$  values are plotted in this figure. The ratio  $285/260$  was used for rapid identification of the peaks. Only two of the peaks gave ratios greater than 1: (1) that centered at tube No. 6 is uric acid with a ratio of 2.6; (2) that centered at tube No. 109 is orotic acid with a ratio of 1.8.

The addition of a mixture of nucleotides<sup>1</sup> effected an excellent reticulocyte rise in the hemoglobin level to normal and a striking reduction in the excretion of orotic acid. The appearance of the erythrocytes became normal and megaloblasts disappeared from the marrow. The child gained weight for the first time in 18 months and lost his apparent retardation in walking, talking, and general activity.

<sup>1</sup> Concentrated from yeast extract by Schwartz Laboratories, Inc., Mt. Vernon, N.Y., containing 110 mg cytosylic acid and 1760 mg uridylic acid per milliliter.

intake was increased. Proof of the identification of these crystals as orotic acid is given elsewhere [7]. Briefly the excreted material corresponded to authentic orotic acid samples with respect to elution characteristics from ion-exchange columns.  $R_F$  values on paper chromatograms

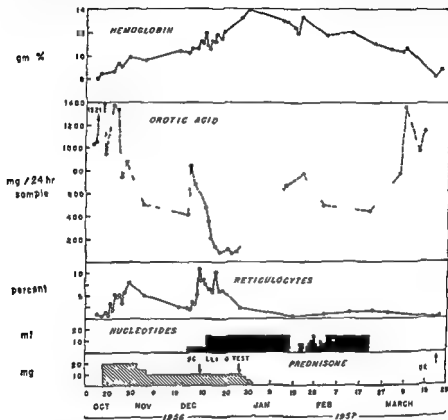


Fig 23-4. Changes in hemoglobin, reticulocytes and urinary orotic acid in a patient with orotic aciduria as influenced by administration of prednisolone and a mixture of nucleotides. Medication is diagrammed in the lowest two segments of the figure. The nucleotide mixture contained uridylic acid 115 mg per ml and cytidylic acid 269 mg per ml.

in several solvent systems, ultraviolet absorption spectrums, melting points of the original material and derivative, and elemental analysis. Determinations were made by fractionation on a Dowex column and ultraviolet spectrophotometric determination of the concentration in the appropriate fractions of eluate (Fig 23-5).

Figure 23-3 also shows the hematologic ineffectiveness of uracil. An attempt was made to observe the effect of uracil on orotic acid excretion, but the technique for collecting 24 hr urine specimens was inadequate at that time. Orotic acid excretion was reduced little, if at all.

separate administration of uridylic and orotidylic acid which might have provided a more definitive localization of the defect

This case also provides an instructive illustration of the negative feed back mechanisms which control the production of intermediates in biosynthetic pathways by regulating either the formation of enzymes or their activity [53]. Such feed back mechanisms have been shown in various microorganisms to control the formation of purine precursors [34] arginine [35] isoleucine [56] and orotic acid [14, 37-39] among

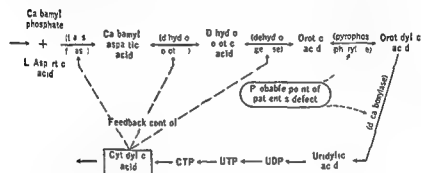


Fig. 23-6. Probable point of metabolic defect and possible sites of feed back control mechanism.

others. Similar mechanisms have been inferred from experiments with avian [60] and mammalian systems [61-62]. The case under discussion seems to be most closely analogous to that described in *Escherichia coli* mutants by Yates and Lardee [63] in which it was found that accumulation of carbamylaspartate, dihydroorotate, and orotate was inhibited by the presence of uracil or cytosine. This inhibition was shown to be the result of the fact that cytidine and especially cytidylic acid are competitive inhibitors of aspartate-carbamyl transferase. The mechanism of inhibition by the cytidylic-uridylic acid mixture of orotic acid accumulation in the patient can only be inferred by analogy, but it seems probable that a control mechanism existed in this patient similar to that described by Yates and Lardee in bacteria. The situation may be summarized as in Fig. 23-6.

Aspartate-carbamyl transferase appears to be the most probable point at which control is exerted. This reaction is favored because it is the first one which is specific for pyrimidine biosynthesis and because it is irreversible. Cytidylic acid is shown in the figure as the most likely controlling material because of its relative specificity as an inhibitor of the transferase, as shown by the work of Yates and Lardee [63]. It should be noted however that Smith and Baker [63] have reported that, in contrast

When prednisolone was then stopped the remission continued but when nucleotides were stopped the patient rapidly relapsed and while in relapse contracted a severe case of varicella which involved the lungs and led to his death. At autopsy no abnormalities were found which might have explained the megaloblastic anemia and oroticaciduria.

As far as has been determined from a search of the literature this case is unique. As indicated above it does not fall into any of the well-defined categories of megaloblastic anemia nor does the megaloblastosis seem to be a secondary phenomenon. While cases of idiopathic vitamin B<sub>12</sub>- and folic acid-refractory anemia have been described [39, 40] the authors have been unable to find a case on record of oroticaciduria or even a note of crystalluria as a feature of megaloblastic anemia.

The recognition of crystalluria in this case was due to the combination of a rather massive excretion of orotic acid and a disinclination of the patient to take much liquid when ill. In other circumstances it might easily have been missed. Nevertheless the urines of 73 other persons have been negative for orotic acid. These included specimens from normal children and adults, both parents and all three siblings of the patient, 19 persons (some in relapse some after therapy) with various types of megaloblastic anemia including intestinal malabsorption syndrome, nutritional megaloblastic anemia and the megaloblastic anemia due to primidone and diphenylhydantoin, as well as Addisonian pernicious anemia, three patients with erythremic myelosis, and patients with a variety of nonmegaloblastic anemias.

## BIOCHEMICAL CONSIDERATIONS

The findings clearly imply a defect in the pyrimidine synthetic pathway *de novo* between orotic acid and uridylic acid (or perhaps cytidylic acid). The region of the block may be further delimited if it is assumed that in man the orotidylic decarboxylase reaction is irreversible, as it is in yeast [16]. Since the equilibrium between orotic acid and orotidylic acid lies far towards the former, a defect in either orotidylic pyrophosphorylase or orotidylic decarboxylase would account for the observed accumulation of orotic acid (Fig. 23.6). There was at least a tenfold preponderance of the orotic acid peak in the urinary chromatogram over the adjacent peaks (Fig. 23.5) and the absorption spectrums of the adjacent peaks showed little resemblance to those of suspected intermediates. Unfortunately an exhaustive characterization of the minor components of the urinary chromatogram was not performed, so that the excretion of small amounts of orotidylic acid or orotidine (which might arise from dephosphorylation of the former) cannot be ruled out with certainty. In any case the excreted materials are probably not strictly relatable in quantity to those occurring at the tissue of origin. The early death of the patient prevented the

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to the bacterial enzyme the aspartate carbamyl transferase from human blood cells is not inhibited by cytidylic acid. Obviously either dihydro-orotase or the dehydrogenase might be the point of control, and uridylic acid UDP, UTP, CTP or other compounds derived from them are candidates for the controlling material. Uridine diphosphate has been shown to control the activity of orotidylic decarboxylase [62] in a mammalian system. Extension of such studies as these will probably allow an eventual evaluation of the relative effectiveness of the members of the pyrimidine biosynthetic system as inhibitors of the various enzymes involved.

## GENETIC CONSIDERATIONS

The evidence that this disease is due to the deficiency of an enzyme has been given above. Since symptoms appeared early in life there is strong presumption that the enzyme deficiency was genetic in origin but since no other members of the family are known to have been affected there is no proof that it is genetic. Nevertheless because of the consanguinity of the parents the opportunity was present for the apparent transmission of a recessive trait in the homozygous state in the propositus (Fig. 23.2). If this is what occurred the heterozygous state is not associated with oroticaciduria nor with anemia or morphologic abnormalities of the mature erythrocyte.

## SUMMARY

1 Oroticaciduria has been described once. It was associated with a severe anemia developing in infancy and was characterized by hypochromic erythrocytes and a megaloblastic marrow. There was some improvement in all features of the disease after the administration of adrenocorticoids and almost complete clearing of the evidence of illness after the administration of a mixture of uridylic and cytidylic acids.

2 Megaloblastosis would be expected as a result of failure in pyrimidine synthesis. Relief by administration of uridylic and cytidylic acid is as might be predicted. The subsequent fall in orotic acid excretion is probably attributable to a negative feed back control mechanism as has been demonstrated in bacterial systems.

3 Orotic acid is an obligatory intermediate in *de novo* pyrimidine synthesis. The excretion of large amounts would indicate a block in metabolic progression to orotidylic acid or to uridylic acid. A lack of either orotidylic pyrophosphorylase or orotidylic decarboxylase would account for the observed accumulation of orotic acid.

4 Recently reduced levels of both enzymes were found in the erythrocytes of both parents and two of the surviving siblings of the deceased propositus [64].

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## Chapter 24

### Beta aminoisobutyricaciduria\*

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*H Eldon Sutton*

The development of paper chromatography provided a powerful tool for the recognition of hitherto undetected substances in biologic fluids. Among the first to exploit the potentials of this technique was Dent, who examined urine samples from many normal and diseased persons and recorded the presence of several previously unrecognized substances which form colored products with ninhydrin [1]. Of the substances that he encountered in a number of samples was one which he first designated as T spot and which was later identified independently by Crumpler et al [2] and by Fink, Henderson, and Fink [3] as  $\beta$  aminoisobutyric acid ( $\alpha$  methyl  $\beta$  alanine). Crumpler et al [2] found that readily detectable amounts of  $\beta$  aminoisobutyric acid (BAIB) occurred persistently in the urine of approximately 48 per cent of normal British subjects. They suggested that the rate of excretion is controlled by genetic factors. On the other hand, Fink et al [3] in addition to finding a small number of normal individuals who were high excretors, found that a large percentage of cancer patients also were high excretors. Figure 24-1 shows photographs of two-dimensional amino acid chromatograms, one from a high BAIB excretor and the other from a low excretor.

Since these early papers, both the genetic basis for BAIB excretion and the association of BAIB excretion with certain types of disease have been amply confirmed. Harris [4] studied a series of families in London and reported that some individuals consistently excrete large amounts of BAIB (70 to 300 mg per day) and that these persons tend to occur in the same families. Other studies have confirmed this. On the other hand, BAIB excretion has also been reported in association with certain neoplastic diseases, particularly leukemia [5-10] and with tuberculosis [11], march hemoglobinuria [12], liver disease [13], lead poisoning [14].

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mongolian idiocy and other types of mental defect [15-17] epilepsy [18] and following heavy irradiation with neutrons and  $\gamma$  rays [19] Several other diseases have been reported with high BAIB excretion but in most cases these have involved too few patients to exclude a chance association with an inherited high excretion of BAIB or else the procedures for identifying BAIB were inadequate Berry [19a] has recently summarized studies of BAIB excretion in a large number of healthy and diseased

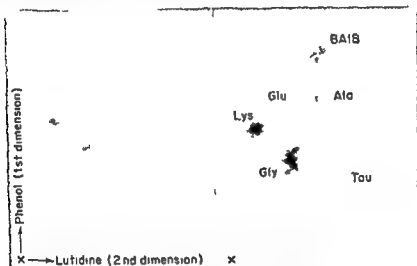


Fig 24.1 Two-dimensional amino acid chromatograms of urine from a low BAIB excretor (left) and from a high BAIB excretor (right). An aliquot of urine containing 50  $\mu$ g creatinine was applied in the lower left corner. Color development was with ninhydrin. Abbreviations: alanine (Ala), glutamine (Glu), glycine (Gly), lysine (Lys) and taurine (Tau).

infants. She found that the high excretion associated with diseases is variable and that in some cases it could be influenced by diet. No evidence has been obtained that inherited BAIB excretion is deleterious.

Although BAIB frequently occurs in high levels in urine, it does not occur in sufficient quantities for detection in other body fluids which have been tested (blood, spinal fluid, saliva). It was not found at appreciable levels in urine samples from 55 primates [10]. On the other hand, Tallan, Moore, and Stein [20] reported barely detectable quantities in the liver, brain, and kidneys of a cat. Its occurrence in appreciable concentrations, however, seems limited to the urine of some human beings.

## METABOLIC REACTIONS INVOLVING BAIB

### Relation to Thymine

In their paper on the identity of BAIB, Fink, Henderson, and Fink [3] recognized that the carbon skeleton of BAIB is present in thymine and

## Chapter 24

### Beta aminoisobutyricaciduria\*

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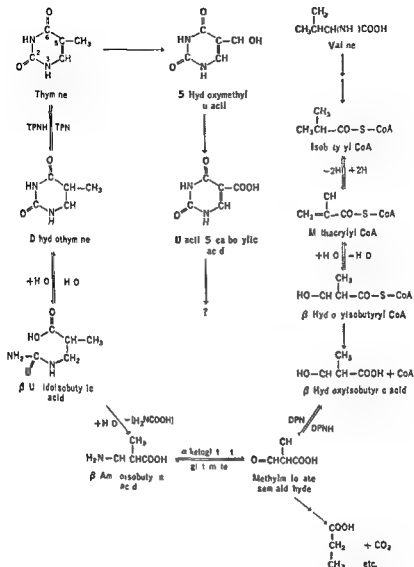


Fig 712 Establishes the metabolic reaction of BAIB as indicated and the

The same enzyme preparation (partially purified) also used  $\beta$ -alanine and  $\gamma$  aminobutyric acid as substrates

### Steric Considerations

Since the  $\alpha$ -carbon of BAIB is asymmetric the question of optical isomerism should be considered in treating the metabolism of BAIB

that BAIB might therefore be a metabolic end product of thymine. Evidence favoring this hypothesis was obtained by feeding large amounts of DNA and RNA to rats. BAIB cannot normally be detected in the urine of rats but on the DNA diet it is excreted. By contrast the RNA diet which does not contain thymine does not cause the excretion of BAIB. The high levels of nucleic acid used in these diets (40 per cent) raised doubts as to the significance of the effect observed but subsequent studies by this same group [21] have confirmed that BAIB can be a metabolite of thymine. Of a variety of pyrimidines injected into rats only thymine and dihydrothymine caused the appearance of BAIB in the urine. Even 5-methylcytosine which has the same carbon skeleton as thymine failed to cause the excretion of BAIB.

Both thymine and dihydrothymine can yield BAIB when incubated with rat liver or kidney slices [22]. Liver is more active in this respect. Dihydrothymine is a much more effective precursor than thymine and thymidine is no better than thymine. No BAIB is formed when these substrates are incubated with a variety of tissues other than liver and kidney. On the other hand liver and kidney from a variety of mammals were shown to form BAIB.

In a subsequent series of papers the Finks and their associates have demonstrated the conversion of thymine by way of dihydrothymine and  $\beta$  uridoisobutyric acid to BAIB as shown in Fig 24.2 [23-24]. An analogous series of reactions was also demonstrated for the conversion of uracil to  $\beta$  alanine [23] and there is evidence that the cleavage of the dihydropyrimidine ring is catalyzed by the same enzyme in both pathways [25]. Canellakis [26] studied these reductive degradation pathways for uracil and thymine and reported that the initial reduction of the pyrimidine ring requires reduced TPN. Grisolia and his associates [27-28] studied the decarbamylation reactions of  $\beta$  ureidopropionic acid and  $\beta$  ureidoisobutyric acid and found that formation of the  $\beta$  amino acids is accompanied by the production of  $\text{NH}_3$  and  $\text{CO}_2$ . However Fink et al [29] using labeled thymine found that the C2 carbon appears in urea. In addition they found that some of the thymine is oxidized at the methyl group indicating an alternate oxidative pathway for thymine degradation. Whether the urea is formed from oxidative degradation or from the carbamate of the reductive pathway is not known. Fink et al [29] were unable to detect 5-methylbarbituric acid which is a breakdown product of thymine in microorganisms.

#### *Relation to Valine*

Coon and his associates have demonstrated the production of BAIB from valine [30-34]. The reactions are indicated in Fig 24.2. Of particular interest is the BAIB-glutamic transaminase reaction studied by Kupiecki and Coon [34]. This conversion of BAIB to methylmalonate semialdehyde provides a means of further catabolism of BAIB regardless of its origin.

mental evidence to support this concept. This mechanism can now be excluded. Crumpler et al [2] reported that the blood levels of BAIB in genetic high excretors were no higher than in the rest of the population and considered this as evidence for a defect in tubular reabsorption. The levels which they found were not reported and it is not clear that they actually detected BAIB in the plasma of any of their subjects. The amount of BAIB circulating in the plasma is far less than other amino acids and paper chromatographic methods which can be used for other amino acids in plasma are inadequate for BAIB either in high or low excretors. Evered [35] was unable to detect BAIB in the plasma by ion exchange chromatography. He computed that the plasma clearance in a high excretor must be at least 85 ml per min indicating little if any tubular reabsorption in these persons. Gartler [36] increased the blood levels of BAIB in nonexcretors artificially by oral administration of thymine and found that the plasma clearance actually exceeded the glomerular filtration rate. Thus it seems likely that BAIB must be classified with those substances which are not reabsorbed at all thereby ruling out a renal mechanism for high excretion both in genetic and nongenetic high excretors.

#### *Rate of Formation of BAIB*

In considering the possibility that increased BAIB excretion results from increased formation from precursors it is necessary to decide which of the two possible pathways—from thymine and from valine—is quantitatively more important. The evidence is meager but favors the thymine pathway. Awapara and Shullenberger [9] found that administration of 1 gm thymine to a variety of patients suffering from neoplastic diseases results in a manyfold increase in BAIB excretion. Similarly Gartler [37] found that ingestion of thymidine, thymine, dihydrothymine or  $\beta$  ureidoisobutyric acid by normal high and low excretors caused increased excretion of BAIB. On the other hand, the oral administration of 500 mg valine to a genetic high excretor and to a low excretor did not result in an increase of BAIB in the urine [37]. This amount of valine is much less than the amount of other amino acids used in tolerance loads and possibly is inadequate to test the ability of valine to serve as a precursor of BAIB. Awapara and Shullenberger [9] also found an increase in BAIB excretion in many of their patients after administration of nitrogen mustard. They interpret this as a consequence of the release of thymine from tissue and suggest that excretion of BAIB may be a useful index of the extent of tissue destruction.

This rate of excretion provides an explanation for the association of high BAIB excretion with certain disease involving increased tissue destruction (e.g. leukemia, march hemoglobinuria). It was early reported that the rate of excretion varied with the clinical state of the patient in



Unfortunately, this has been largely ignored. BAIB from the urine of genetic excretors is levorotatory [2] but this in itself does not indicate which pathway is responsible for the production of excessive amounts of BAIB. Complicating this picture is the observation by Fink, Henderson and Fink [21] that only 15 per cent of a racemic mixture of BAIB injected intraperitoneally into rats is recovered in the urine. This would suggest that both forms can be metabolized. Since the BAIB glutamic transaminase appears to be insensitive to the methyl group reacting either with  $\beta$  alanine or with  $\alpha$  methyl  $\beta$  alanine (BAIB) it is quite possible that it is also insensitive to the steric configuration of the  $\alpha$ -carbon. In the case of valine catabolism and undoubtedly in the case of thymine catabolism only one isomer would be formed in vivo.

#### *Relation to Other Imino Acids*

Although BAIB conforms chemically to the definition of an amino acid it should not be grouped metabolically with the other amino acids. It has never been isolated from a protein nor is it known to participate in peptide bond formation. As will be discussed later it probably does not compete with other amino acids in transport reactions. The amino nitrogen does enter the nitrogen pool of the amino acids by means of the transaminase reaction.

### CAUSES OF EXCESS BAIB EXCRETION

The nature of the metabolic defect which results in BAIB excretion has not been established either in genetic excretors or in persons suffering from various diseases. Indeed there is no a priori reason for supposing that the cause is the same in the two cases. On the basis of the known metabolic reactions of BAIB it is possible to suggest several mechanisms by which BAIB excretion might be increased. It should be kept in mind that there is virtually no information concerning the quantitative importance of the pathways which have been established nor for that matter is it often known which direction predominates in these frequently reversible reactions.

An increased excretion of a metabolite has one of three causes (1) increased formation of that metabolite from its precursors (usually because some alternate pathway of the precursor is blocked), (2) decreased capacity to metabolize the product with the result that the blood levels increase and the substance appears in the urine (3) decreased ability to reabsorb the metabolite from the glomerular filtrate.

#### *Role of Renal Mechanisms*

A renal mechanism has frequently been postulated to account for  $\beta$  aminoisobutyricaciduria but there has never actually been experi-

In the absence of additional experimental evidence it is obviously impossible to decide which of the two pathways from thymine or valine is quantitatively important in the excretion of BAIB. The significant evidence will most probably come from tracer studies rather than from loading experiments in which unphysiologic levels of metabolites are achieved. A point which must also be considered is that in conditions of excessive tissue destruction BAIB excretion occurs in persons who are not genetic excretors. This implies that the ability to metabolize BAIB is limited in such persons also but at a level which permits them to handle normal turnover without appreciable excretion of BAIB. Such a delicate balance is unusual but certainly not impossible.

## GENETICS OF BAIB EXCRETION

### *Detection Methods and the Problem of Classification*

It is impossible to discuss the genetics of BAIB excretion without referring to methods of assay. BAIB was discovered by means of paper chromatography which has remained the principal means of detection. Ion-exchange methods as applied by Evered [31] are the most accurate available at present but genetic studies depend upon the assay of large numbers of samples and the ion-exchange methods are very time consuming. Of necessity the method of choice has been paper chromatography in spite of the relatively large error inherent in its use as a quantitative tool. Furthermore there are several ninhydrin reacting substances which occasionally appear in small quantities in positions on the chromatogram very close to BAIB and it is not always possible on a single chromatogram to distinguish these substances. For these reasons the trace amounts of BAIB frequently reported are apt to reflect substances other than BAIB.

The difficulties imposed by analytic error are apparent when one attempts to assign each individual to a particular category of excretion. In the previous discussions the terms high and low excretor have been used as if the distribution of excretion rates were discontinuous. It would indeed be convenient if individuals could be assigned to nonoverlapping categories since conventional Mendelian analysis is based on attributes rather than on continuous variables. The evidence on this point is contradictory. Some find evidence of bimodality [46] while others find no evidence for bimodality [43]. In populations where the frequency of high excretors is low a very large population sample would be required in order to demonstrate a significant separation between the two groups. However even in populations where the frequency of high excretors is high the evidence for a clear separation between high and low excretors is not convincing. Cartler [37] using a paper electrophoretic technique for estimating BAIB has obtained results which he considers to be

contrast with the relatively constant rate of excretion of normal high excretors [3]. High BAIB excretion is frequently observed in hospitalized patients particularly in small children suffering from obscure metabolic disorders [38]. In the absence of other family information it is probably best to consider these patients as having excessive tissue destruction or at least altered DNA metabolism.

### *Metabolic Disposal of BAIB*

There is certainly no reason to suppose that genetic high excretors have increased tissue destruction. It is conceivable that their DNA metabolism may differ from that of low excretors but Gartler [36, 37] has provided evidence that the difference is in the handling of metabolites subsequent to the reduction of thymine. This is most clearly indicated by the results of oral and intravenous administration of DL-BAIB. Low excretors retain most of the BAIB while high excretors dispose of it in the urine. Since the conversion of  $\beta$  ureidoisobutyric acid to BAIB is essentially irreversible there is no reason to implicate reactions of the thymine pathway prior to the actual formation of BAIB.

It seems necessary to postulate a block in the conversion of BAIB to methylmalonate semialdehyde or in the further metabolism of the latter to propionate etc. If the transaminase reaction is blocked then the catabolism of valine should have little influence on the excretion of BAIB. On the other hand if the block occurs subsequent to methylmalonate semialdehyde and assuming this pathway to be the principal means of valine breakdown, then valine should also contribute to the BAIB excreted.

It is informative to consider the quantities of the various possible precursors which would be required to account for the urinary BAIB in a high excretor. If an excretion rate of 200 mg BAIB per day is assumed as a reasonable value for a high excretor then 240 mg thymine, 470 mg thymidine, 1.04 gm DNA or 227 mg valine would have to be converted with 100 per cent efficiency to urinary BAIB to account for this rate. If the DNA is considered to represent tissue breakdown then approximately 400 to 800 gm fresh tissue would have to be destroyed per day to produce this much DNA (assuming concentrations of 25 to 50  $\mu$ g DNA phosphorus per 100 mg fresh tissue [39]). Such a turnover rate perhaps is not unreasonable [40]. It is quite possible that even if thymine is responsible for all the BAIB produced it does not all come from completed DNA. Valine is an essential amino acid and arises entirely from the diet. The average daily per capita consumption of valine is about 5 gm [41] of which only about 10 per cent is needed to maintain positive nitrogen balance [42]. Thus a diversion of about 5 per cent of the valine into BAIB would account for the amount of BAIB excreted even in high excretors.

amounts of BAIB behaved as a recessive trait. The only mating of high excretor with high excretor produced three high-excretor offspring.

A subsequent study of an Italian population by Calchi Novati et al [45] using the same procedures as those of Harris revealed a frequency of 40 high excretors in a population of 711 individuals over 4 years of age. Again the analysis of family material indicated consistency with the hypothesis that high excretors are homozygous for a recessive gene. The only mating of high excretor with high excretor produced a single high excretor offspring.

Gartler [47] studied a series of New York families using 30 mg per gm creatinine as the dividing line. There were no matings of high excretor with high excretor but the distribution of offspring types in the other matings was consistent with the inheritance of high excretion as a recessive trait.

Grouchy and Sutton [48] studied the inheritance of BAIB excretion in 17 families of Chinese or Japanese extraction. This population is particularly useful since the frequency of high excretors is very much higher in it than in Caucasian populations (Fig 24.2). In the absence of evidence for a distinct antimode statistical techniques were applied which are valid for multigenic inheritance. These showed a high correlation among siblings and between offspring and parents confirming the essentially genetic character of this trait. Although neither the type of segregation within families nor the type of progeny produced by the various matings was entirely compatible with a simple two-allele hypothesis the evidence did not exclude the possibility that a major portion of the genetic variation could be accounted for by a simple two-allele Mendelian system.

Gartler, Firschein and Kraus [49] reported a series of family studies on a Black Carib population which has a high frequency of BAIB excretors. They chose as their dividing line the value

$$\log (\text{O.D. BAIB} / \text{O.D. glycine}) = 1.40$$

where O.D. = the optical density of the amino acid spots measured on paper chromatograms.

The value 1.40 is the point at which an antimode occurred in this population. The results again were in general agreement with the hypothesis that high excretion results from homozygosity for a recessive gene. In this instance there were 5 matings of high excretor with high excretor producing 24 high-excretor offspring and 2 low-excretor offspring. The latter 2 low-excretor offspring are inconsistent with the simple 2 allele system but the true family relationships may not have been ascertained.

In summarizing the foregoing genetic studies perhaps the best conclusion is that the major variation in BAIB excretion is under the control of a very few genes possibly a single pair of alleles and that high excretion results from homozygosity for a recessive allele. There still appear to be

superior to those obtained by the chromatographic methods. As yet no family studies with this method have been reported.

### *Environment Sex Diet and Age*

Before proper genetic studies can be undertaken it is necessary to evaluate the environmental factors which may alter the trait under study. Experiments have already been described in which the BAIB excretion is altered by ingestion of large amounts of thymine, dihydrothymine or  $\beta$ -ureidorsobutyric acid. Such situations are not likely to arise outside a laboratory, however, and all the other evidence favors the view that diet is ordinarily ineffective in changing the level of BAIB excretion. On the assumption that thymine is the precursor of BAIB one study was carried out by placing two individuals—one a high excretor and the other a low excretor, on a meat free diet for 1 week [38]. Although certain other features of the excretion pattern changed the BAIB excretion remained constant and quite different for each individual. In another study of the excretion of BAIB within families, no correlation was found between husbands and wives but there was a significant correlation between parents and offspring [43]. This is good evidence that BAIB excretion is not readily influenced by usual dietary variations. It seems best to consider that excretion of BAIB is the result of endogenous processes not readily influenced by either quantity or quality of food. Other environmental factors have not been investigated for their influence on BAIB excretion but in view of the individual consistency in excretion rates there is little reason to suppose that such factors play a significant role.

Many metabolites vary considerably with age or sex but in the case of BAIB these too appear to be unimportant. None of the studies in which sex differences were investigated has reported a significant difference [4, 43-47]. For the most part no age differences have been reported [4, 43, 46] although several investigators have found increased levels of BAIB among young children (less than 4 or 5 years of age) [10, 44, 46, 47]. The importance of this increase in young children is not known but it suggests caution in the interpretation genetic or otherwise of the excretion levels of young children.

### *Genetic Control*

The first study of the genetic control of BAIB excretion was that of Harris [4] in England. He selected as his dividing line between high and low excretors the amount of BAIB which reacted on paper with ninhydrin to give a spot equal in intensity to the alanine spot of the same urine sample. This amount was estimated to be about 70 mg per gm creatinine. Of the 345 persons examined 33 (9.6 per cent) were high excretors by this criterion. In the family studies the excretion of large

amounts of BAIB behaved as a recessive trait. The only mating of high excretor with high excretor produced three high-excretor offspring.

A subsequent study of an Italian population by Calchi Novati et al [45] using the same procedures as those of Harris revealed a frequency of 40 high excretors in a population of 711 individuals over 4 years of age. Again the analysis of family material indicated consistency with the hypothesis that high excretors are homozygous for a recessive gene. The only mating of high excretor with high excretor produced a single high excretor offspring.

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In summarizing the foregoing genetic studies perhaps the best conclusion is that the major variation in BAIB excretion is under the control of a very few genes, possibly a single pair of alleles, and that high excretion results from homozygosity for a recessive allele. There still appear to be

other factors either genetic or nongenetic or both, which can influence the amount of BAIB excreted. As new assay techniques become available and the experimental error can be reduced the nature of the genetic factors should also become much clearer.

### RACIAL VARIATION IN BAIB EXCRETION

As with many other inherited traits the frequency of the genes controlling BAIB excretion varies considerably in different populations. This

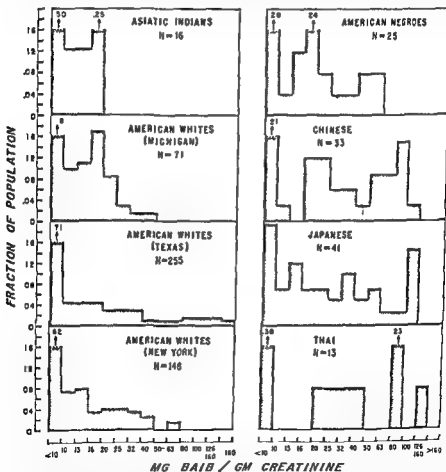


Fig. 24-3. Distribution of excretion rates of BAIB in various populations. References are given in Table 24-1.

was recognized first during the comparison of the amino acid excretion patterns of a small group of Chinese with that of Americans of European extraction [48]. Instead of the rare high excretor found among Europeans the Chinese were found to have a very high frequency of high excretors.

This difference among populations has been confirmed and extended and the results of some of the studies are summarized in Fig 24-3. Another means of expressing these differences is in the proportion of individuals whose level of excretion is greater than some value. In Table 24-1 are shown the results expressed in this manner with studies of some additional populations whose published values were not expressed in a way which permitted their inclusion in Fig 24-3. It is apparent that the highest excretors occur among the Orientals and among the American Indians with intermediate values for Negroes. Allison, Blumberg, and

TABLE 24-1 FREQUENCIES OF INDIVIDUALS EXCRETING BAIB AT LEVELS GREATER THAN 30 MG PER GM CREATININE IN VARIOUS POPULATIONS

| Population        | Frequency of excretors | % of persons | Reference |
|-------------------|------------------------|--------------|-----------|
| American white    |                        |              |           |
| Michigan          | 0.03                   | 71           | [33]      |
| Texas             | 0.078                  | 205          | [1]       |
| New York          | 0.115                  | 148          | [4]       |
| Asiatic Indians   | 0.00                   | 16           | [38]      |
| Negroes           |                        |              |           |
| Michigan          | 0.90                   | 23           | [34]      |
| Black Caribs      | 0.116                  | 285          | [10]      |
| New York          | 0.15                   | 38           | [10]      |
| Chinese           | 0.45                   | 31           | [38]      |
| Japanese          | 0.415                  | 41           | [33]      |
| Thai              | 0.46                   | 11           | [38]      |
| Ap chos (Arizona) | 0.416                  | 111          | [10]      |

Gartler [40] have also reported high values for the Athabaskan-speaking Indians of Alaska but found that the Alaskan Eskimos are intermediate between the Indians and whites.

The existence of such genetic polymorphism among all the populations studied can be interpreted as indicating a very early origin of the (two) alleles with the heterozygous individuals possibly enjoying some selective advantage over their homozygous neighbors. The possibility of independent mutations at this locus cannot be excluded, of course. The nature of the selective factors is unknown.

## SUMMARY

1.  $\beta$ -Aminoisobutyric acid (BAIB) occurs in the urine of some persons in amounts up to 200 to 300 mg per day either as an inherited trait or as a consequence of some disease process.

2. Enzymes are present in animal tissues which form BAIB from thymine and from valine but present evidence is not conclusive as to the



actual origin of urinary BAIB. In some disease states the increased BAIB excretion probably reflects excessive tissue destruction with concomitant high DNA catabolism.

3. BAIB is not reabsorbed by the renal tubules either in low or high excretors of BAIB. The failure of genetic low excretors to excrete ingested BAIB while high excretors do excrete ingested BAIB is evidence that a block exists in the further metabolism of BAIB. This may be either in the transamination to methylmalonate semialdehyde or in subsequent steps.

4. The evidence for dimorphism in the excretion rates of BAIB is not clear cut although recently developed techniques may resolve some of the earlier reported discrepancies. Nevertheless the principal genetic variation seems to be largely explained on the basis of a two-allele system with high excretors being homozygous for a recessive gene. There still remains variation either genetic or nongenetic which is not explained by such a system.

5. Sex and diet do not appear to influence the excretion of BAIB. Very young children are reported to be high excretors more often than adults but age does not appear to affect the excretion rate among persons over 5 years of age. The frequencies of persons who excrete large amounts of BAIB vary with the population under study. The lowest frequencies are found in white populations and the highest in Mongolians and American Indians with Negroes intermediate.

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## Part Seven

### Diseases Primarily Manifest as Abnormalities in Metal Metabolism

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## Chapter 25

### Wilson's Disease\*

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*Alexander G. Bearn*

It is quite certain that if this book had been conceived 25 years ago it would have been comfortably slimmer and would not have included a chapter on Wilson's disease. The view would have been almost unanimously held that the disease could not be considered either inherited or metabolic. Although during the past 25 years the nature of the disease has become greatly clarified, the basic metabolic defect underlying its cause remains obscure.

Wilson's disease (hepatolenticular degeneration) is a relatively rare disease occurring predominantly in young people. It is characterized by cirrhosis of the liver and degenerative changes in the brain, particularly in the basal ganglia. The greenish brown Kayser-Fleischer rings seen in the periphery of the cornea are pathognomonic of Wilson's disease. Genetic studies have revealed that the condition is inherited in an autosomal recessive fashion.

#### CLINICAL FEATURES

In 1912 Kinross Wilson published his monograph entitled "Progressive Lenticular Degeneration: A Familial Nervous Disease Associated with Cirrhosis of the Liver." [1] While still a young man Wilson had under his care a patient who died after a long illness characterized by muscular rigidity, tremor, and forced grimacing. Cirrhosis of the liver and gross degenerative changes in the lenticular nucleus of the brain were found at autopsy. On searching the literature, Wilson found other cases which were clinically similar. Six years earlier Cowers, in a paper entitled "Tetynoid Chorea Associated with Cirrhosis of the Liver," described

The author is indebted to Professor L. S. Penrose for his helpful discussions concerning the interpretation of the genetic data and to Professor Charles H. Rodeck and Dr. James L. Cerman III for their assistance in the preparation of the manuscript.

two patients whose symptoms were similar to those of Wilson's patient [2]. A review of the literature at that time disclosed that the disease had been seen sporadically in the past but had escaped systematic study. The first case of recognized Wilson's disease was probably described by Frerichs in his classic monograph on liver disease in 1861 [3]. The patient, a young boy, suffered from severe liver disease associated with violent tremors and convulsions and died at the age of 10. An autopsy revealed cirrhosis of the liver. The age of the patient and the nature of his symptoms make the diagnosis of Wilson's disease although unproved highly likely.

The clinical characteristics of Wilson's disease may be so unique that in many instances the diagnosis is not in doubt. Unfortunately the "typical case" is becoming less frequent as understanding of the varied clinical manifestations of the disease increases.

### *Lenticular Degeneration*

This form of the disease is the one seen by Wilson and overemphasized by subsequent observers. In fact, classic lenticular degeneration is a severe but relatively uncommon form of the disease which occurs predominantly in young adults. Spasticity, rigidity, dysarthria, and dysphagia are the common presenting symptoms. Tremor is usually less pronounced and clinical evidence of hepatic disease is minimal or absent although as in all varieties of Wilson's disease pathologic evidence of cirrhosis is almost invariable. Acute unexplained febrile episodes were recognized by Wilson in some of his patients and may usher in the terminal phase of the disease.

### *Pseudosclerosis*

Unnecessary and frequently acrimonious nosologic debate obscured the existence of this variety of hepatolenticular degeneration during the early part of this century. It is now clear that what has been known as pseudosclerosis of Westphal [4] is a variant of Wilson's disease in which tremor is usually the major disabling symptom, while rigidity and spasticity are less marked. Symptoms referable to hepatic dysfunction are uncommon but pathologic evidence of cirrhosis can invariably be found and impairment of Bromsulfalein excretion can frequently be demonstrated [5]. Other biochemical evidence of disturbed liver function is usually slight.

### *Kayser Fleischer Ring*

Despite the brilliance of Wilson's clinical acumen the only pathognomonic sign of the disease escaped him. Ten years before the publication of Wilson's paper Kayser had observed a greenish yellow ring at the limbus of the cornea in a patient diagnosed as having multiple sclerosis. A year later Fleischer reported a corneal ring as an integral part of a

neurologic disease associated with cirrhosis of the liver. In a series of papers [6-8] Fleischer proposed the concept that the changes in the eye, brain, and liver were all due to a common metabolic cause. The Kayser-Fleischer ring is still rightly regarded as the single most important diagnostic sign of the disease. The ring is not always complete and is usually most marked at the superior and inferior aspects of the cornea. Although with experience most Kayser-Fleischer rings can be seen by inspection of the cornea, its presence can be definitely excluded only by a slit lamp examination. In a very few patients careful examination of the eye will reveal a *Sonnenblumenkatarakt*. Similar cataracts are known to follow the intraocular localization of a foreign body containing copper.

### *Cirrhosis of the Liver*

The hepatic form of Wilson's disease, in which there is evidence of liver abnormality with minor or absent neurologic signs, has been recognized as a clinical variant for many years [9]. Its frequency and significance have recently become increasingly appreciated [10]. In a series of 32 cases collected at the Rockefeller Institute between 1951 and 1958, 8 (25 per cent) could be classified as belonging to this group. It seems likely that a significant but unknown proportion of patients with juvenile cirrhosis actually suffer from Wilson's disease. No patient with juvenile cirrhosis has been satisfactorily examined unless the presence of Kayser-Fleischer rings has been diligently sought and the biochemical tests necessary for the diagnosis of Wilson's disease have been performed. The cirrhosis is similar pathologically to that seen in the pseudosclerotic variety of the disease but is generally more severe. Signs of portal hypertension can frequently be demonstrated. In some patients with Wilson's disease the presenting symptom is massive hemorrhage due to rupture of esophageal varices. Hepatic coma may usher in the terminal phase. Ascites rarely occurs except in the later stages of hepatic decompensation.

### *Other Clinical Variants*

A sizable proportion of patients with Wilson's disease may present with symptoms suggesting schizophrenia; others may develop less pronounced abnormalities in behavior or personality. Tragic ignorance of this form of the disease has resulted in needless shock therapy and unnecessary detention in psychiatric hospitals.

Occasionally patients may develop epileptic seizures, usually of the Jacksonian type. Hemiplegia is not so rare as is commonly supposed. A curious comatose state may persist for several weeks and does not necessarily herald an immediate fatal outcome.

### *Etiology*

Two hypotheses are current concerning the probable pathogenetic mechanism of Wilson's disease. The first and presently more favored



hypothesis regards the disease as a primary disturbance in copper metabolism. The second ascribes the disease to a more generalized disorder of tissue protein metabolism. Neither of these two hypotheses is entirely satisfactory and both philosophy and common sense suggest that the final view will incorporate features of both. For purposes of clarity in exposition these two hypotheses will be dealt with separately and the defects of each emphasized.

## NORMAL METABOLISM OF COPPER

Copper is an ubiquitous element. Under normal circumstances the average individual consumes between 2.5 and 5.0 mg copper per day. Foods with high copper content include liver, shellfish, nuts, and chocolate. Cow's milk usually contains only a small amount of copper and has been used successfully to induce copper deficiency in certain animals. In most foodstuffs copper is bound principally to proteins and amino acids. Copper may be present in significant quantities in drinking water particularly if the pipes are copper lined.

### *Ceruloplasmin Copper*

Copper in the serum is bound to the plasma proteins. Approximately 98 per cent of the circulating serum copper in man is bound to an  $\alpha_2$ -globulin. Purification of the  $\alpha_2$ -globulin fraction has resulted in the isolation of a copper protein which, because of its blue color, is called ceruloplasmin.

Ceruloplasmin has a molecular weight of about 151,000 and contains eight copper atoms per molecule [11, 12]. Holmberg and Laurell [13, 14] have described some of its properties. The protein is an oxidase with many properties in common with plant laccases. Among the variety of compounds toward which it exhibits oxidase activity are biologically important substances such as epinephrine and serotonin, as well as synthetic substrates [15]. The greatest oxidase activity has been demonstrated using as substrate the dye, paraphenylenediamine. The enzymatic activity may be accelerated by low concentrations of a variety of monovalent anions including chloride, bromide, and acetate. Higher concentrations of these may inhibit. Some polyvalent ions, phosphate and citrate, for example, may inhibit the enzymatic reaction and cause a prolonged induction period. Whether ceruloplasmin exhibits enzyme function *in vivo* is uncertain.

Procedures designed to dissociate copper from ceruloplasmin have resulted in loss of enzymatic activity. It has been demonstrated by the use of  $\text{Cu}^{64}$  that under appropriate conditions half of the eight copper atoms of each ceruloplasmin molecule can exchange with ionic copper [16]. This will take place only if the protein first is reduced to its colorless form. Ceruloplasmin *in vivo* may, under certain circumstances, exchange

its copper with copper absorbed from the gastrointestinal tract [17]. If this mechanism can be demonstrated to occur under physiologic conditions ceruloplasmin would perform a role in copper transport similar to that of transferrin in iron transport. It is also possible that ceruloplasmin is a storage protein. Since a gross deficit of ceruloplasmin has been demonstrated in a healthy carrier of the abnormal gene of hepatolenticular degeneration it is unlikely that its enzymatic function is vital to the organism. The relationship of ceruloplasmin to mental disease [18] remains too speculative for further comment. It is clear that the relationship, if any, is both tenuous and indirect [19].

**Ceruloplasmin Determination** Serum ceruloplasmin can be measured in a variety of ways. Holmberg and Laurell originally demonstrated a positive correlation between the copper content of the serum and its oxidase activity using paraphenylenediamine as a substrate. The oxidase activity can be measured directly in a Warburg apparatus and recorded in terms of micromoles of  $O_2$  uptake per milliliter per hour. Alternatively the oxidase activity can be measured colorimetrically. Ravin has outlined a rapid method for ceruloplasmin determination using this principle [20]. Other methods for estimating this protein depend upon the blue color. Measurement of the optical density before and after destruction of the blue color by ascorbic acid or cyanide is an index of the concentration of ceruloplasmin [21]. Ceruloplasmin can also be measured immunologically [22]. This of course is dependent upon the availability of a pure preparation of ceruloplasmin which can be used as an antigen. In 1958 Broman [23] reported the existence of multiple ceruloplasmins in normal plasma with similar enzymatic properties. The main component accounts for approximately 80 per cent of the total ceruloplasmin. The heterogeneity of ceruloplasmin observed by Broman and recently confirmed by Saks Kortrik [23a] may have important implications in the understanding of the mechanism of gene action in Wilson's disease.

#### *Nonceruloplasmin Copper*

It can be shown by salt fractionation and more precisely by electrophoretic techniques that the nonceruloplasmin copper is bound to serum albumin [24]. The physical and chemical characteristics of the copper albumin complex have been extensively studied [25]. In general it may be stated that the main copper binding sites of the albumin molecule are the imidazole groups but some weaker binding also occurs at the carboxyl group. The extent of the binding depends on the pH and the competitive effect of other anions in solution. Heating to approximately 100 C does not disturb the ability of protein to form complexes with copper in contrast to its effect on protein anion complexes. The essential requirements for binding appear to be the presence of appropriate residues and favorable electrostatic conditions of the molecule.

hypothesis regards the disease as a primary disturbance in copper metabolism. The second ascribes the disease to a more generalized disorder of tissue protein metabolism. Neither of these two hypotheses is entirely satisfactory, and both philosophy and common sense suggest that the final view will incorporate features of both. For purposes of clarity in exposition these two hypotheses will be dealt with separately and the defects of each emphasized.

## NORMAL METABOLISM OF COPPER

Copper is an ubiquitous element. Under normal circumstances the average individual consumes between 2.5 and 5.0 mg copper per day. Foods with high copper content include liver, shellfish, nuts, and chocolate. Cow's milk usually contains only a small amount of copper and has been used successfully to induce copper deficiency in certain animals. In most foodstuffs copper is bound principally to proteins and amino acids. Copper may be present in significant quantities in drinking water, particularly if the pipes are copper lined.

### *Ceruloplasmin Copper*

Copper in the serum is bound to the plasma proteins. Approximately 98 per cent of the circulating serum copper in man is bound to an  $\alpha_2$ -globulin. Purification of the  $\alpha_2$ -globulin fraction has resulted in the isolation of a copper protein which, because of its blue color, is called ceruloplasmin.

Ceruloplasmin has a molecular weight of about 151,000 and contains eight copper atoms per molecule [11, 12]. Holmberg and Laurell [13, 14] have described some of its properties. The protein is an oxidase with many properties in common with plant laccases. Among the variety of compounds toward which it exhibits oxidase activity are biologically important substances such as epinephrine and serotonin, as well as synthetic substrates [15]. The greatest oxidase activity has been demonstrated using as substrate the dye paraphenylenediamine. The enzymatic activity may be accelerated by low concentrations of a variety of monovalent anions including chloride, bromide, and acetate; higher concentrations of these may inhibit. Some polyvalent ions, phosphate and citrate, for example, may inhibit the enzymatic reaction and cause a prolonged induction period. Whether ceruloplasmin exhibits enzyme function *in vivo* is uncertain.

Procedures designed to dissociate copper from ceruloplasmin have resulted in loss of enzymatic activity. It has been demonstrated by the use of  $\text{Cu}^{2+}$  that under appropriate conditions half of the eight copper atoms of each ceruloplasmin molecule can exchange with ionic copper [16]. This will take place only if the protein first is reduced to its colorless form. Ceruloplasmin *in vivo* may, under certain circumstances, exchange

copper [30] The liver, central nervous system and kidney have the highest copper content (Table 25-2) The muscles and bones because of their total mass contain over 50 per cent of the total body copper Curiously, the fetal liver contains about ten times the amount of copper found in an adult liver The copper content of the liver of a child reaches the level found in a young adult by the age of 10

Several copper proteins have been isolated from the liver Mann and Keilin isolated a colorless copper protein from the liver of the ox [31]

TABLE 25-2 MEAN COPPER CONTENT OF VARIOUS ORGANS IN NORMAL SUBJECTS AND PATIENTS WITH WILSON'S DISEASE

| Organ         | Normal subjects | Patients with Wilson's disease |
|---------------|-----------------|--------------------------------|
| Liver         | 0.5             | 10.0 (5.0-20.0)                |
| Kidney        | 0.3             | 1.0 (0.5-2.0)                  |
| Basal ganglia | 0.6             | 3.0 (2.0-8.0)                  |
| Cortex        |                 |                                |
| White         | 0.5             | 5.0 (3.0-10.0)                 |
| Gray          | 0.4             | 6.0 (4.0-8.0)                  |

All values expressed as mg per 100 gm wet weight

This protein was not crystallized and accounted for only a part of the total copper present in the liver Mohamed and Greenberg isolated an apparently crystalline copper protein from horse liver The protein was blue contained 0.30 to 0.40 per cent copper and had an estimated molecular weight of 30,000 to 40,000 This protein has been named hepatocuprein unlike ceruloplasmin it does not exhibit any oxidase activity [32]

The nature of the copper proteins of normal brain (cerebrocuprein) has been extensively studied by Huntington Porter [33-35] Thus far three copper-containing fractions of normal brain have been isolated and their properties described Fraction I is extracted with acetate buffer at pH 4.5 fraction II is obtained by extraction of the residue from fraction I with water at pH 3.5 at very low ionic strength the final residue fraction is designated fraction III From fraction I has been isolated a green copper-containing protein named cerebrocuprein I This protein contains 0.2 to 0.3 per cent copper and appears 60 per cent homogeneous by ultracentrifugal and electrophoretic techniques It has a molecular weight of approximately 30,000 to 40,000 Cerebrocuprein I exhibits two rather singular properties Copper is not released from cerebrocuprein I by dialysis at pH 3.9 and approximately 10 per cent of its copper reacts directly with diethyldithiocarbamate It is not yet known with certainty whether this direct reacting moiety of normal cerebrocuprein I is of physiological importance or whether it is an artifact Copper is widely di-

Ionic copper reacts with diethyldithiocarbamate to form a characteristic yellow color. Copper in ceruloplasmin will not react with this reagent unless the copper is first released from the ceruloplasmin. The copper which is bound to albumin reacts directly with this reagent and permits the detection and quantitation of the albumin bound copper. This fraction has been designated the "direct reacting copper" by Cartwright and his associates [26]. It seems probable that although the total amount of albumin copper does not normally greatly exceed 5 per cent of the total serum copper, it performs the essential task of transporting copper from the intestinal tract to the various tissues of the body and is metabolically the most active fraction of the serum copper.

### *Erythrocuprein*

Copper is a normal component of adult red cells where its function is unknown. At least 80 per cent of the copper in erythrocytes is present

TABLE 25-1 A COMPARISON OF THE PHYSICAL AND CHEMICAL PROPERTIES OF ERYTHROCUPREIN AND CERULOPLASMIN

| Property                     | Erythrocuprein          | Ceruloplasmin   |
|------------------------------|-------------------------|-----------------|
| Color                        | Nearly colorless        | Blue            |
| Absorption maxima            | 655 m $\mu$ 765 m $\mu$ | 605 280 m $\mu$ |
| Molecular weight             | 33,000                  | 151,000         |
| Percentage of copper         | 0.37-0.34               | 0.32-0.34       |
| Atoms of copper per molecule | 2                       | 8               |
| Isoelectric point            | 5.3                     | 4.4             |

SOURCE: Adapted from C. I. Cartwright et al. See references [27-29].

in the form of erythrocuprein, a nearly colorless protein recently isolated from human red cells by Cartwright and his colleagues [27-29]. A comparison of some of the physical properties of erythrocuprein and ceruloplasmin is shown in Table 25-1.

In copper deficient animals a severe anemia develops which is morphologically similar to the anemia of iron deficiency. The erythrocytes of the copper deficient pig have a shortened survival time but when transfused into a normal pig they have approximately the normal life span. Erythrocytes transfused from a normal to a copper deficient pig also survive normally. Such evidence suggests that the shortened survival time of erythrocytes in copper-deficient animals is not due to an extracorporeal abnormality. Rapid exchange can take place between the serum copper and the red cell copper both *in vivo* and *in vitro* [27].

### *Tissue Copper*

It is hardly surprising that copper is widely distributed in all body tissues. The body of a normal adult contains approximately 150 mg

1915 when Glazebrook reported a patient with Wilson's disease whose liver and brain at autopsy revealed an increased copper concentration and whose serum copper was considered elevated [41] (In view of subsequent findings the increased serum copper reported may possibly have been in error) In all events the observations of Glazebrook proved to have catalytic consequences and a bewildering spate of papers on copper metabolism ensued The next major event was the chance finding by Mandelbrote and his coworkers [42] that a patient suffering from Wilson's disease excreted an increased quantity of copper in the urine Numerous observers have now amply confirmed both the increased tissue copper and the high urinary copper excretion Considerably more disagreement has developed in regard to the serum copper concentration in Wilson's disease Although initial observers reported both normal and elevated levels it was subsequently shown that the levels were almost invariably decreased [43] Recently the wheel has turned full circle and several patients with unequivocal Wilson's disease have now been observed in whom the serum copper is unquestionably normal [44-45] whether this is normal ceruloplasmin will be discussed more fully later

### *Serum Ceruloplasmin*

Almost simultaneously in different laboratories and with different methods diminished ceruloplasmin levels were found in patients with Wilson's disease The first method used was based on its enzymatic activity [43] as described by Holmberg and Laurell and the second more elegant method based on quantitative immunologic procedures in which antiserum had been prepared against crystalline ceruloplasmin [22] A colorimetric procedure based on its oxidase properties has also been employed

Although the majority of patients with Wilson's disease have a diminished serum ceruloplasmin level the degree of depression varies widely In some individuals essentially no ceruloplasmin can be detected In others the level is depressed to about 25 per cent of its normal value (Fig 23-1) Several subjects with Wilson's disease have been reported in whom the ceruloplasmin levels were within the normal range Whether the ceruloplasmin present is precisely the same molecule which exists in normal subjects requires further study

In some patients with Wilson's disease as in normal subjects an increase in the ceruloplasmin concentration can be achieved by the administration of estrogens (Fig 23-2) [46] This effect is most marked in those in whom the ceruloplasmin level is least depressed As might be anticipated in those patients in whom little or no ceruloplasmin can be detected no measurable increase in the ceruloplasmin level occurs In some patients a pronounced cupuremia may follow the administration of estrogens [47a]

tributed in all parts of the brain. The selective deposition of copper in the basal ganglia has been unduly stressed. Vogel [35a] has recently demonstrated an increased copper content of the brain of goldfish when reared in an environment in which the ionic copper is experimentally increased.

Ceruloplasmin in trace amounts has been found in the liver and the kidney [36]. The physiologic significance of this observation if any is uncertain.

### *Secretions*

Copper is present in trace amounts in most of the body fluids including saliva, milk, tears, and sweat. The urine in the absence of significant proteinuria contains only small amounts of copper; the daily 24 hr urine excretion rarely exceeding 0.1 mg per day. The form in which copper is present in normal urine is uncertain; only part is freely dialyzable.

### *Physiologic Variations in Copper Metabolism*

The significance of the alterations in copper metabolism in Wilson's disease cannot be appreciated unless the normal physiologic variations are understood.

Ceruloplasmin increases under a variety of physiologic conditions. It may be increased in pregnancy, infections, thyrotoxicosis, cancer, and cirrhosis of the liver. It is frequently decreased in the newborn and in sprue, nephrosis, anemia (in infants), and malnutrition (in infants). It is almost invariably decreased in Wilson's disease. The administration of estrogens to normal subjects increases the ceruloplasmin level [37].

## ABNORMALITIES OF COPPER METABOLISM IN WILSON'S DISEASE

### *Historical*

A number of early German workers implicated various heavy metals including copper as possible etiologic agents in Wilson's disease. In 1913, only 2 years after Wilson's classic paper, Rumpel reported an increased silver and copper content of the liver and kidneys of patients who had died from pseudosclerosis [38]. In retrospect, it appears that these patients were almost certainly suffering from unrecognized Wilson's disease. Nine years later, Siemerling and Oloff, on the basis of the similarity of the eye cataracts in Wilson's disease to those following injury from a copper-containing foreign body, made the bold suggestion that copper might be deposited in the liver and brain in this disease as well as in the eye and might be directly responsible for the disease [39].

Because of technical difficulties, the possible role of heavy metals in the etiology of the disease remained confused until 1930, when Haurowitz convincingly demonstrated an increased copper content of the liver and brain [40]. Despite this unequivocal clue, the subject lay dormant until

1945 when Glazebrook reported a patient with Wilson's disease whose liver and brain at autopsy revealed an increased copper concentration and whose serum copper was considered elevated [41] (In view of subsequent findings the increased serum copper reported may possibly have been in error.) In all events the observations of Glazebrook proved to have catalytic consequences and a bewildering spate of papers on copper metabolism ensued. The next major event was the chance finding by Mandelbrote and his coworkers [42] that a patient suffering from Wilson's disease excreted an increased quantity of copper in the urine. Numerous observers have now amply confirmed both the increased tissue copper and the high urinary copper excretion. Considerably more disagreement has developed in regard to the serum copper concentration in Wilson's disease. Although initial observers reported both normal and elevated levels it was subsequently shown that the levels were almost invariably decreased [43]. Recently the wheel has turned full circle and several patients with unequivocal Wilson's disease have now been observed in whom the serum copper is unquestionably normal [44-45] whether this is normal ceruloplasmin will be discussed more fully later.

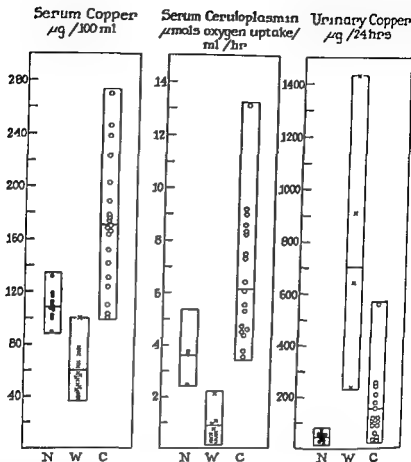
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N Normals  
W Wilson's Disease  
C = Cirrhosis

Fig 25-1 Comparison of amounts of serum copper, serum ceruloplasmin, and urinary copper in normal subject, cirrhotic patients, and patients with Wilson's disease. Horizontal lines represent mean values. Note the one patient with Wilson's disease in whom a normal ceruloplasmin level occurred. (Reproduced from A G Bearn [69].)

### Serum Copper

Early in the course of investigations on Wilson's disease it was found that the serum copper level was higher than would have been expected from the ceruloplasmin levels observed. Since there was no evidence that ceruloplasmin in Wilson's disease contained more copper per molecule, it seemed probable that the nonceruloplasmin copper was increased. Observations by Cartwright have now established that the direct reacting copper is elevated in Wilson's disease (Fig 25-3). It is possible

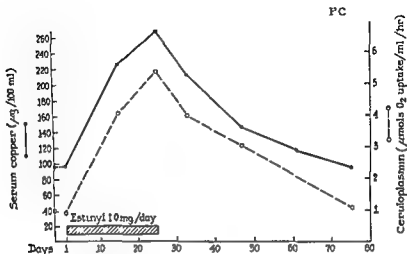


Fig 25.2 Effect of estinyl on the serum ceruloplasmin of a patient with Wilson's disease (Reproduced from J G Beermann [1961] with permission of the Honorary Editor of the Royal Society of Medicine)

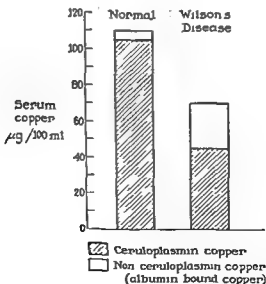


Fig 25.3 Note (1) decrease in total serum copper (2) decrease in serum ceruloplasmin (3) increase in directly reacting copper (albumin bound copper) in Wilson's disease

that the increased concentration of the direct reacting copper reflects the mechanism by which excess copper is deposited in the tissue

### *Urinary Copper Excretion*

Although an increased copper excretion is usually considered to be one of the cardinal biochemical abnormalities in Wilson's disease it should be appreciated that a moderate increase in urinary copper excretion can occur in cirrhosis of the liver particularly of the biliary type. Urinary copper excretion estimations are, therefore, unreliable in excluding Wilson's disease as a cause for juvenile cirrhosis. Fortunately this important clinical differential diagnosis can usually be settled by a determination of the level of serum ceruloplasmin (Fig. 25.1).

The extent of the increased urinary copper excretion in Wilson's disease varies considerably. In general those individuals with the shortest duration of clinical disease tend to have the more nearly normal levels, those in whom the disease has been present for a long time may excrete as much as 1.5 mg copper daily. In individual patients it is usually possible to observe a slow but progressive increase in the urinary excretion of copper over a period of years. Thus far no patient with clear clinical evidence of Wilson's disease has been seen in whom the urinary copper excretion was completely normal.

Under standard dietary conditions day-to-day fluctuations in the urinary excretion of copper are relatively slight and are largely independent of the urinary volume and the dietary copper intake [47]. In contrast to the insensitivity of the urinary excretion of copper to a twenty-five fold increase in copper intake a ten fold increase in the dietary protein intake results in an approximately two fold increase in the urinary excretion of copper. In normal subjects trivial and inconstant increases in copper excretion are observed following a similar increase in the protein intake. The increased copper excretion which follows the augmented protein intake is paralleled by the increased amino acid excretion. It is tempting to ascribe in part the increased copper excretion to the capacity of many amino acids to chelate copper. However quantitative considerations compel one to qualify such an interpretation for chelation implies a necessary molar relationship between the quantity of amino acids and the quantity of copper excreted.

### *Tissue Copper*

The intrahepatic distribution of copper in Wilson's disease has been recently studied by Uzman using a histologic method [48]. Rubeanic acid forms a black precipitate with copper possibly because of the formation of the imido internal salt of rubeanic acid. Under neutral or slightly alkaline conditions this reaction has a reported sensitivity of 0.006  $\mu\text{g}$  copper. The original method using rubeanic acid suffered from certain

drawbacks. The modification recently introduced appears more satisfactory although attention to detail is still imperative if reproducible results are to be obtained. A marked variation in the copper content of various liver lobules has been described.

The intracellular distribution of copper can be arbitrarily divided into three stages. At first a fine diffuse distribution of copper is present throughout the cytoplasm of the liver cell. Later copper is concentrated around one pole of the nucleus. In the heavily involved lobules the granular copper deposits near the nucleus are coarse but become finer as the periphery of the cell is approached. Copper is not found in the Kupffer cells of the liver. Examination of the histologic appearance of the liver at various stages of the disease suggests that the copper deposition precedes the cirrhotic process. It seems clear that the liver cells have a particular affinity for copper. Whether this is because of the presence of specific abnormal liver proteins with a high affinity for copper or because of the binding of copper to normal cellular constituents will be discussed in detail later.

In the brain copper is distributed widely. No single area appears exempt. In some instances copper is deposited in large quantities in the cerebral cortex, but usually the basal ganglia bear the brunt of the disease. Although the details of the histologic localization of copper in the brain remain uncertain its presence in glial rather than nerve cells has been reported. The neurons however show deterioration [49].

The Kayser Fleischer ring has been examined for the presence of heavy metals ever since the early studies of Siemerling and Orloff. Recently the histochemical and electron microscopic studies of Uzman have clarified a rather confused subject [50]. It has now been demonstrated by two groups of workers that the copper content of the substantia propria may be increased without causing any cloudiness of the cornea or any noticeable color change. The copper in the substantia propria is alcohol insoluble but easily removed by dilute acids or chelating agents such as Verene. In Descemet's membrane on the other hand the copper is localized as a fine granular deposit arranged in two parallel zones close to the endothelial surface of the membrane. The characteristic brown or gray green color of the Kayser Fleischer ring is not directly due to the deposition of copper in Descemet's membrane. The presence of dense layers of copper depositions separated by intervening clear zones provides an ideal physical system for the scattering and reflection of incident light on the cornea and gives rise to the characteristic appearance of the Kayser Fleischer ring.

#### *Dynamic Aspects of Copper Metabolism*

Thus far this account of the abnormalities of copper metabolism in Wilson's disease has been entirely restricted to the realm of descriptive

biology, it seems appropriate now to consider some of its dynamic aspects. This problem has been clarified by the use of radioactive isotopes. Copper<sup>64</sup> has a half life of only 12.88 hr which limits the time during which studies on man can be performed. However, another isotope of copper, Cu<sup>67</sup>, with a considerably longer half life exists; if it becomes available, it will enable prolonged studies to be performed to supplement the observations obtained using Cu<sup>64</sup>.

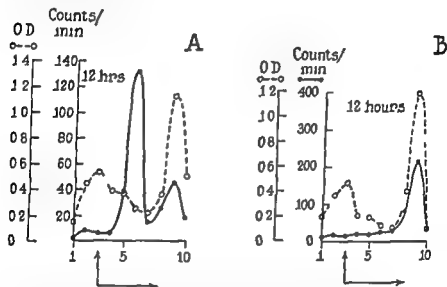


Fig. 25-4. Distribution of radioactive copper in the electrophoretically separated fractions of the serum of control subjects (A) and a patient with Wilson's disease (B) 12 hrs following oral administration of Cu<sup>64</sup>. (Adapted from I. G. Bearn et al. [21].)

A characteristic and reproducible series of curves is obtained after the intravenous administration of radioactive copper to normal subjects [21, 22] (Fig. 25-4). An initial rapid fall in serum labeled copper is followed by a rise. This is succeeded by a slow decline of radioactivity. In patients with Wilson's disease the initial decline of radioactivity is slower than in normal subjects and no secondary rise is observed. Electrophoretic separation of serum and salt fractionation at various intervals after administration of the isotope have shown that immediately following the administration of Cu<sup>64</sup> the radioactivity is bound to serum albumin and no radioactivity is detected in any of the other serum fractions. During the normal secondary rise the radioactivity is associated with the ceruloplasmin and little or no labeled copper is found in association with the albumin. In patients with Wilson's disease as in normal subjects the Cu<sup>64</sup> is first associated with serum albumin but it remains bound to albumin at a time when in normal persons the Cu<sup>64</sup> is associated with ceruloplasmin (Fig. 25-5).

The tentative explanation of these findings is that in both normal subjects and patients with Wilson's disease copper is transported on the serum albumin to various parts of the body. Some copper is transported to the site of ceruloplasmin synthesis where provided the capacity to synthesize ceruloplasmin is normal it becomes incorporated into newly synthesized ceruloplasmin. Since impaired ceruloplasmin synthesis is a usual feature of Wilson's disease the copper transported to the liver is not utilized in this way; it is deposited at various tissue sites and remains

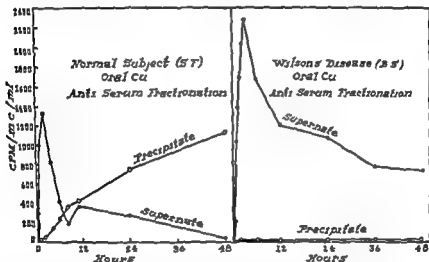


Fig. 23-5 Radioactivity in fractions of plasma produced by addition of rabbit anti-human ceruloplasmin serum. The values on the ordinate refer to counts per minute per milliliter of radio-copper ingested appearing in fractions derived from 1 ml plasma (Reproduced from S. I. Burch et al. [5]).

bound to the albumin in quantities greater than in normal subjects. The copper albumin complex is a relatively loose bond; the copper can easily be dissociated from the protein moiety. The increased quantity of copper in the urine in Wilson's disease is no longer anomalous, for although the total circulating copper is diminished, the copper bound to serum albumin is increased and copper is excreted in the urine. An asymptomatic normal sibling in whom ceruloplasmin was deficient handled intravenous  $\text{Cu}^{64}$  similarly to patients with clinical Wilson's disease [38].

Recently Sass Kortsak has studied the fate of intravenous  $\text{Cu}^{64}$  in a patient with Wilson's disease in whom the ceruloplasmin level was normal [45]. No radioactivity could be demonstrated in association with the ceruloplasmin, and the  $\text{Cu}^{64}$  remained associated with the serum albumin as in those patients with Wilson's disease in whom ceruloplasmin was depressed. Whether this failure of incorporation of  $\text{Cu}^{64}$  into cerulo-

plasma is evidence of a failure to exchange the radioactive copper with the nonradioactive copper or whether as seems more likely, it is due to an alteration in the structure of ceruloplasmin, remains to be determined.

Since the total body copper is increased in Wilson's disease and because the urinary excretion is elevated it is clear that an increased quantity of dietary copper must be absorbed through the intestinal tract. Balance studies have tended to indicate that increased absorption does indeed occur as does a decreased excretion of copper through the intestinal tract. The use of radioactive copper has enabled the increased absorption of copper to be demonstrated clearly [52].

Uptake of  $\text{Cu}^{64}$  by erythrocytes (in erythrocytuprein) of patients with Wilson's disease is similar to that of normal subjects. This emphasizes the rather specific nature of the ceruloplasmin defect in this condition [53]. An explanation for the hemolytic anemia found in some patients with Wilson's disease is not readily deduced from the studies on copper metabolism of the red cell [54].

## RENAL ABNORMALITIES IN WILSON'S DISEASE

In 1948 Uzman and Denny Brown reported an increased amino aciduria in patients with Wilson's disease [55]. Dent in the previous year had also observed an increased excretion of amino acids in Wilson's disease [56]. In the succeeding 10 years it has become clear that the renal lesion in Wilson's disease is extremely diffuse and affects many aspects of renal function [57].

### *Renal Hemodynamics*

In most cases of Wilson's disease there is a marked and consistent reduction of renal plasma flow and a distinctly decreased glomerular filtration rate. In general the degree of impairment parallels the severity and duration of overt disease. In some instances albuminuria can be demonstrated. There may be impairment in urine concentrating ability.

### *Tubular Reabsorptive Mechanisms*

The tubular secretory capacity is usually diminished as judged by impairment of the  $Tm_{\text{PAH}}$ . Associated with this defective function a diminished effect of probenecid in eliciting a further reduction of  $Tm_{\text{PAH}}$  can be demonstrated.

### *Amino Acids*

Impairment of renal tubular reabsorptive activities in Wilson's disease has been thoroughly demonstrated. The excessive aminoaciduria can be attributed to a defective renal reabsorption since qualitatively and quantitatively the amino acids in the serum are not elevated. It is known

that the various amino acids are not reabsorbed in the tubules by a common process thus it is of interest to examine the distribution of the amino acids excreted in the urine in patients with Wilson's disease. It should be emphasized that increased aminoaciduria is not a constant feature of the disease and is usually present only in those patients in whom the disease is of long standing. In general it appears that the largest excretion relative to normal subjects occurs with threonine and cystine excretion of which may be elevated twentyfold [58]. Indeed the excretion of cystine in Wilson's disease may exceed that found in patients

TABLE 25-3 URINARY AMINO ACID EXCRETION IN WILSON'S DISEASE

| Diminished         | Normal             | Increased<br>(X normal) |            |           | Excretion<br>of amino<br>acids not<br>found in<br>normal<br>urine |
|--------------------|--------------------|-------------------------|------------|-----------|---|
|                    |                    | 2-4                     | 5-10       | >10       |   |
| Taurine            | Amino acid<br>acid | Histidine               | Serine     | Threonine | Proline   |
| 1 Methyl histidine | Methionine         | Ornithine               | Glycine    | Cystine   | Citrulline  |
| 3 Methyl histidine | Isoleucine         | Phenylalanine           | Asparagine |           |   |
|                    | Leucine            |                         | Glutamine  |           |   |
|                    | Arginine           |                         | Valine     |           |   |
|                    |                    |                         | Tyrosine   |           |   |
|                    |                    |                         | Lysine     |           |   |

SOURCE: W. H. Stein et al. [58].

with cystinuria. It is of interest that patients with Wilson's disease usually do not form cystine stones. The excretion of other amino acids in relation to the normal excretion is illustrated in Table 25-3.

The urinary excretion of amino acids in Wilson's disease can vary markedly depending upon the state of the disease and the composition of the diet.

In addition to an increased excretion of free amino acids there is an increased excretion of amino acids in the form of conjugated linkages from which the free acids are liberated by hydrolysis with acid. These bound amino acids excreted in quantities about double those of the normal urine thus represent a smaller proportional elevation than that found for free amino acids [53].

In recent years Uzman has emphasized that one of the cardinal abnormalities in Wilson's disease is the excretion of specific oligopeptides [59]. These are thought to be formed by an abnormal proteolytic enzyme in the tissues and to cause an intracellular accumulation of oligopeptides



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In most cases of Wilson's disease there is a marked and consistent reduction of renal plasma flow and a distinctly decreased glomerular filtration rate. In general the degree of impairment parallels the severity and duration of overt disease. In some instances albuminuria can be demonstrated. There may be impairment in urine concentrating ability.

### *Tubular Reabsorptive Mechanisms*

The tubular secretory capacity is usually diminished as judged by impairment of the  $Tm_{\text{PAH}}$ . Associated with this defective function is a diminished effect of probenecid in eliciting a further reduction of  $Tm_{\text{PAH}}$  which can be demonstrated.

### *Amino Acids*

Impairment of renal tubular reabsorptive activities in Wilson's disease has been thoroughly demonstrated. The excessive aminoaciduria can be attributed to a defective renal reabsorption, since qualitatively and quantitatively the amino acids in the serum are not elevated. It is known

that the various amino acids are not reabsorbed in the tubules by a common process thus it is of interest to examine the distribution of the amino acids excreted in the urine in patients with Wilson's disease. It should be emphasized that increased aminoaciduria is not a constant feature of the disease and is usually present only in those patients in whom the disease is of long standing. In general it appears that the largest excretion relative to normal subjects occurs with threonine and cystine excretion of which may be elevated twentyfold [58]. Indeed the excretion of cystine in Wilson's disease may exceed that found in patients

TABLE 25-3 URINARY AMINO ACID EXCRETION IN WILSON'S DISEASE

| Diminished         | Normal               | Increased<br>( $\times$ normal) |            |           | Excretion<br>of amino<br>acids not<br>found in<br>normal<br>urine |
|--------------------|----------------------|---------------------------------|------------|-----------|---|
|                    |                      | 2-4                             | 5-10       | >10       |   |
| Taurine            | Amino adipic<br>acid | Histidine                       | Serine     | Threonine | Proline   |
| 1-Methyl histidine | Methionine           | Ornithine                       | Glycine    | Cystine   | Citrulline  |
| 3-Methyl histidine | Isoleucine           | Phenylalanine                   | Asparagine |           |   |
|                    | Leucine              |                                 | Glutamine  |           |   |
|                    | Arginine             |                                 | Valine     |           |   |
|                    |                      |                                 | Tyrosine   |           |   |
|                    |                      |                                 | Lysine     |           |   |

SOURCE: W. H. Stein et al. [58].

with cystinuria. It is of interest that patients with Wilson's disease usually do not form cystine stones. The excretion of other amino acids in relation to the normal excretion is illustrated in Table 25-3.

The urinary excretion of amino acids in Wilson's disease can vary markedly depending upon the state of the disease and the composition of the diet.

In addition to an increased excretion of free amino acids there is an increased excretion of amino acids in the form of conjugated linkages from which the free acids are liberated by hydrolysis with acid. These bound amino acids excreted in quantities about double those of the normal urine thus represent a smaller proportional elevation than that found for free amino acids [58].

In recent years Uzman has emphasized that one of the cardinal abnormalities in Wilson's disease is the excretion of specific oligopeptides [59]. These are thought to be formed by an abnormal proteolytic enzyme in the tissues and to cause an intracellular accumulation of oligopeptides

within the tissues. Some of the excreted peptides compete with the amino acids for reabsorption by the proximal renal tubules and, because the copper peptide complex is absorbed preferentially, an aminoaciduria results. In discussions of this hypothesis it should be recalled that the liberation of amino acids on acid hydrolysis of urine is not evidence per se of the existence of peptides. At least 75 per cent of the amino acids liberated by acid hydrolysis of human urine derives from compounds other than peptides [60]. A considerable proportion of the increase in the amounts of glutamic and aspartic acids after acid hydrolysis arises from the decomposition of glutamine and asparagine. Other compounds include such substances as hippuric acid, phenacetylglutamine and tyrosine O sulfate. However, small quantities of peptides undoubtedly exist in normal urine and may be presumed to exist in the urine of patients with Wilson's disease. Unequivocal demonstration of their presence will be awaited with interest.

### *Glucose*

Glycosuria may be present in some patients with Wilson's disease. It is significant that although in many patients spontaneous glycosuria may be absent or minimal, the maximum tubular capacity to reabsorb glucose is frequently substantially reduced [57]. These observations emphasize that a failure to find an increased excretion of glucose does not preclude a considerable defect in the capacity of the renal tubules to transport glucose across the tubular epithelium.

### *Bicarbonate*

A tendency to excrete an alkaline urine in patients with Wilson's disease has been described and is associated with the renal excretion of bicarbonates at plasma levels that would ordinarily demand complete reabsorption of bicarbonate by the renal tubules.

### *Uric Acid*

In 1954 Bishop and coworkers reported a low serum uric acid associated with an increased urinary uric acid excretion in a brother and sister with Wilson's disease [61]. Subsequent investigators have confirmed these findings, and renal clearance studies have demonstrated that, as originally postulated, the diminished reabsorption of filtered urate accounts for both the increased urinary excretion and the low serum urate levels observed. The capacity of probenecid further to increase the  $C_{\text{rate}}$  to  $C_{\text{cr}}$  diminished as the tubular transport systems for urate deteriorated with progression of the disease [57]. The low serum uric acid level is so constant a feature of Wilson's disease that a serum uric acid determination should be performed in all cases of cirrhosis of unexplained origin and in all obscure diseases of the central nerv-

ous system in which disturbances of the function of the basal ganglia are prominent

### Phosphate

A low serum inorganic phosphate level occurs in a high proportion of patients with Wilson's disease [67]. Renal clearance studies have revealed an even higher proportion of patients in whom the  $C_{Cr}$  is increased. Thus



Fig 25-6 Radiograph illustrating fracture head of the right radius showing no evidence of bone union (Reproduced from A G Bearn [66])

a failure of normal tubular transport of phosphate is a common feature of the disease and may be in part responsible for the occasional appearance of clinical osteomalacia and spontaneous fractures (Fig 25-6) [62-63]

### Calcium

Hypocalcaemia occurs in many patients with Wilson's disease and is also related in part to the duration of the overt disease [63a]

## THE ABNORMALITY IN WILSON'S DISEASE

In the preceding pages the various abnormalities in copper metabolism and renal function have been discussed and the two current hypotheses of the pathogenesis of the disease briefly mentioned. These two hypotheses will now be discussed in detail.

### Abnormality of Copper Metabolism

In its simplest form this hypothesis suggests that the primary effect of the abnormal gene is to diminish the synthesis of normal ceruloplasmin

and conceivably of other closely related proteins. In a way which is not understood this leads to an increased absorption of copper from the intestinal tract, and this in turn to an excessive deposition of copper in the tissues with consequent functional and structural alterations. Although the copper content of many organs is increased certain tissues such as the basal ganglia of the brain, liver cells and Descemet's membrane of the cornea appear to be selected preferentially. The differential deposition of copper in various tissues could be ascribed to the variation of copper binding capacity of normal proteins and related substances in different tissues. No postulation that these tissue proteins are abnormal would be necessary. Thus the disturbed liver function leading eventually to cirrhosis of the liver, the cerebral damage, and the disturbance in renal function are all ultimately consequent upon the effect of copper on a variety of enzyme systems throughout the body. The decreased ceruloplasmin level observed in some individuals who are heterozygous indicates that even in single dose the abnormal gene may have an observable metabolic defect but without causing manifest clinical disease.

When this hypothesis is stated in this simple way many experimental observations do not fit. (1) There is no correlation between the depression of ceruloplasmin synthesis and the amount of copper absorbed. (2) Experimental administration of copper to a variety of animals has failed to mimic the disease. (3) Some patients with Wilson's disease have a serum ceruloplasmin level which is quantitatively normal. (The direct reacting copper level is usually elevated in these individuals, this probably reflects the increased body stores of copper.) (4) There is relatively little correlation between the increased amount of copper excreted in the urine and the clinical improvement which may follow the use of British anti-lewisite (BAL) and penicillamine. Many of these objections do not by themselves invalidate this hypothesis but serve to emphasize its considerable defects.

A reasonable hypothesis for the renal lesion ascribes the progressive deterioration in renal function to the march of copper to sites where it can interfere with enzyme systems responsible for the transport of materials across epithelial membranes. Such a hypothesis gains some support from observations on poisoning with other divalent metals in which most of the damage falls, as it does in Wilson's disease, on the proximal renal tubules. It seems plausible that the renal abnormalities can be ascribed to a disturbance in copper metabolism, and are not a direct consequence of the abnormal gene on renal function.

#### *Abnormality of Peptide Metabolism*

An alternative hypothesis has been postulated by Uzman and outlined by him in a series of papers [69 64 65]. This hypothesis ascribes the primary effects of the abnormal gene to an abnormal protein which has

an increased capacity for binding ionic copper. This protein is present in the liver (and presumably in every tissue in which the copper content is increased). The increased affinity of copper for certain tissues blocks the formation of ceruloplasmin, thus the diminished ceruloplasmin level is a secondary phenomenon and is due to the diversion of copper to the tissues. In addition the abnormal protein metabolism in the tissues is said to give rise to specific oligopeptides which compete with amino acids and uric acid for reabsorption in the proximal renal tubules, causing aminoaciduria and uricosuria. Cirrhosis of the liver is ascribed to a nutritional deficiency.

This hypothesis, currently less favored than the first, also contains some unsatisfactory features. Quantitative considerations make it extremely improbable that the renal loss of amino acids could give rise to hepatic cirrhosis, and indeed many cases of Wilson's disease have been reported in which no aminoaciduria is present but in whom a diminished ceruloplasmin is evident [58-66]. The difficulties and uncertainties in the peptide hypothesis for the urinary abnormalities have been outlined in an earlier section and have been dealt with at length by Walshe [67]. According to this hypothesis it would be anticipated that intravenously administered radioactive copper would disappear from the circulation more rapidly rather than more slowly if the tissue proteins had an increased affinity for copper. Thus it can be seen that neither hypothesis can be regarded as more than tentative; the problem will be resolved not by debate but by the collection of more data.

## GENETICS

In his original monograph Kimmel Wilson pointed out that familial incidence is one of the striking features of the disease. Although he considered the possibility that the disease was inherited, the familial incidence was attributed to environmental rather than genetic causes [1]. This view, with some notable dissent [68], was held until relatively recently.

Study of a large number of patients with Wilson's disease has permitted a genetic analysis [69-70]. Inspection of the pedigrees immediately confirmed the familial concentration observed by Wilson and in addition disclosed that many of those afflicted were the offspring of consanguineous unions (Table 25-4).

This remarkably high consanguinity rate is good evidence that the disease is inherited in an autosomal recessive fashion (Table 25-4). Calculations based on the incidence of Wilson's disease in siblings when the parents are unaffected are in accord with this hypothesis [70]. The method of calculation used is variously known as Hoggan's factorial method or the a priori method of Bernstein.

TABLE 25-4 THE CONSANGUINITY RATE IN 30 FAMILIES WITH WILSON'S DISEASE

| No of sibships | Consanguinity rate in parents of patients with Wilson's disease |            |                |            |           |            |
|----------------|---|------------|----------------|------------|-----------|------------|
|                | First cousins   |            | Second cousins |            | Unrelated |            |
|                | No  | Percentage | No             | Percentage | No        | Percentage |
| 10             | 11  | 36.7       | 3              | 10.0       | 16        | 53.3       |

SOURCE Adapted from A. G. Bearn [70a]

*Sex Incidence*

The disease is probably slightly more common in males than females. Most series of cases show a slight excess in males which because of the small numbers is statistically insignificant. In our series 21 of 32 patients were male.

*Age of Onset*

The age of onset of Wilson's disease is variable and does not differ in the two sexes (Table 25.5). The apparent excess of Jewish males when the

TABLE 25.5 THE ESTIMATED AGE OF ONSET AND GEOGRAPHIC ORIGIN OF 32 PATIENTS WITH WILSON'S DISEASE

| Age of onset yr | Consanguinity | Sex  |        | Geographic origin         |               |       |       | Total |
|-----------------|---------------|------|--------|---------------------------|---------------|-------|-------|-------|
|                 |               | Male | Female | Eastern European (Jewish) | Mediterranean | Negro | Other |       |
| 10-24           | R             | 5    | 3      | 3                         | 3             | 0     | 2     | 11    |
|                 | U             | 5    | 3      | 1                         | 2             | 1     | 4     | 8     |
|                 | T             | 10   | 6      | 4                         | 5             | 1     | 6     | 16    |
| 25-30           | R             | 4    | 4      | 1                         | 1             | 1     | 0     | 8     |
|                 | U             | 7    | 1      | 4                         | 2             | 0     | 2     | 8     |
|                 | T             | 11   | 5      | 10                        | 3             | 1     | 2     | 16    |

Includes one sibling pair

Note: R = related U = unrelated T = total

SOURCE Adapted from A. G. Bearn [70a]

disease has a late onset raises the problem of genetic heterogeneity. The possibility exists that the allele causing disease in this group of patients is different from the usual allele. A modifying gene which delays the onset of the disease could also be responsible for the late onset in these patients.

### *Racial Incidence*

Of the 30 families in New York City in whom individuals with Wilson's disease were discovered 13 (43 per cent) were Jewish who came from the border of Russia and Poland within a radius of approximately 100 miles. If a mutation occurred in this population the breeding structure would favor the formation of many homozygotes. Seven of the 30 families (23 per cent) were Italians who came from Sicily and the southernmost tip of Italy. The breeding structure in this isolated area would also tend to produce individuals homozygous for the abnormal gene. Nearly all the remainder of the patients of this series came from various parts of Europe. Two of the 30 families (7%) were Negroes. The disease has also been recorded in Chinese, Malaysians, Japanese and Eskimos [70b]. Whether the original mutations which occurred in Eastern Poland and Sicily were identical is not clear. In interpreting these data it must be recalled that these patients were largely collected from New York City and that their racial distribution reflects to some extent the geographic origins of the population of New York.

### *Gene Frequency*

Estimations of gene frequency are fraught with considerable hazard. One of the essential assumptions, namely, that the disease is genetically homogeneous, may be unjustified. Another difficulty encountered if the method devised by Dahlberg [71] is used is the uncertainty of the estimate of the frequency of first cousin marriage in the population from which the sample is drawn. Few precise data are available concerning the estimate of the first cousin consanguinity rate in the populations of Eastern Poland or Sicily 50 years ago. To be sure the figure of 1/1000 estimated by Julia Bell [72] for England is far too low and would give too low an estimate of the gene frequency. If accurate data on the consanguinity rate were known it would be possible to give a rough estimate of the gene frequency in the original populations in Eastern Poland and Sicily. It is impossible, however, to determine the gene frequency in America with any accuracy. As a very rough estimate, if the consanguinity rate in America is assumed to be 1/1000, the calculated gene frequency would be approximately 1/2000, with a disease incidence of 1/400000. It should be emphasized that such calculations are almost valueless in a rare recessive disease in which the condition may have arisen by mutation in a very small community in which consanguineous unions were very numerous.

### *Detection of Carriers*

The detection of asymptomatic carriers of deleterious genetic traits is being attempted in many heritable disorders. Related to this problem



but frequently more difficult to demonstrate is the important question of whether the heterozygote possesses any increased biologic fitness over either homozygote [73]. Unfortunately, with a disease as rare as Wilson's disease it would be virtually impossible to demonstrate any heterozygous advantage.

If the gene product were known with certainty, then its estimation should enable a differentiation between the three genetic groups—homozygous normal, homozygous affected and heterozygous—although some degree of overlap might be expected. Ceruloplasmin depression is a relatively constant biochemical abnormality in patients with Wilson's disease; has been found in some heterozygotes, and not in others [70, 74, 75]. In addition some asymptomatic siblings without Kayser-Fleischer rings have had a low level of ceruloplasmin and were found unable to incorporate  $\text{Cu}^{++}$  into ceruloplasmin [46]. A preliminary study of 10 heterozygotes with normal ceruloplasmin levels has demonstrated less incorporation of ingested  $\text{Cu}^{++}$  into ceruloplasmin than normal [76]. The possibility that aminoaciduria could be detected in asymptomatic siblings of patients with Wilson's disease has been investigated. The first reports indicated that aminoaciduria could be detected in relatives of those affected [51]. Subsequent work has failed to confirm the early findings [47].

There is evidence that the disease in certain families conforms to a distinctive clinical and biochemical pattern. In any one sibship the age of onset, the predominant clinical symptoms, and the biochemical abnormalities may be remarkably similar. In some families only the hepatic form of the disease is present, in others the liver injury is minimal and neurologic symptoms are prominent. The possibility cannot be dismissed that these two clinical syndromes are due to different alleles which may not even be at the same locus [70].

Muller has recognized two categories of gene products which he has called amorphs and neomorphs. The first is associated with an absence of the gene product and the latter with the formation of a new and altered gene product. Wilson's disease may prove to be an example of a disease which is amorphic (usually hypomorphic) but which may occasionally prove to be neomorphic.

## TREATMENT

Until some of the biochemical features outlined in the preceding pages became delineated the treatment of Wilson's disease was entirely symptomatic. On the assumption that some if not all of the symptoms in Wilson's disease may be related to the increased total body copper measures designed to decrease the tissue copper stores might be expected to be therapeutically beneficial.

It is not possible to reduce the copper content of the diet below 1.0 mg per day without considerable caloric restriction. The ingestion of carbo

resins and potassium sulfide has been advocated to reduce copper absorption

In addition to diminishing the copper intake efforts should be made to achieve a large and persistent increase in urinary copper excretion. A variety of agents which chelate copper have been used with success. BAL introduced by Denny Brown [77] has proved a valuable agent in increasing the urinary excretion of copper. It suffers from the disadvantage that it must be suspended in peanut oil and administered by intramuscular injection. Local induration or abscesses may occur at the site of injection. Moreover, toxic reactions to BAL are not uncommon. These include high fever, faintness, nausea and vomiting. Intravenous disodium versenate is an effective cupruric agent, but the necessity for intravenous injection of this compound precludes its continued use.

The introduction of penicillamine ( $\beta\beta$  dimethylcysteine) in 1956 by Walshe was a landmark in the development of therapeutic measures for increasing the urinary excretion of copper [78]. This compound, which can be given by mouth, is in the practical dosage range a much more effective cupruric agent than BAL, and its administration is not accompanied by so many side reactions. Toxic reactions may occur, however, and resemble those seen in penicillin sensitivity. Fever, various drug rashes, and leukopenia have been observed. Pharmacologic studies of the two enantiomorphs—D penicillamine and L-penicillamine—indicate not only that the D form is the more effective in protecting rats against mercury poisoning, but that the L-form has serious toxicity for the animals [79]. Such studies do not argue strongly in favor of clinical use of the D-optical isomer rather than the DL-mixture, but indicate the need for a comparative therapeutic evaluation.

One of the difficulties encountered in the treatment of Wilson's disease has been the tendency of patients to become resistant to the cupruric agent, and a prolonged cupruric effect may be difficult to achieve. Presumably, the copper easily removed is that recently deposited, whereas the fraction of copper corresponding to cerebrocuprein II is less easily chelated. None of the chelating agents consistently lowers the serum ceruloplasmin level. The excretion of amino acids or other substances absorbed by the proximal renal tubules and excreted in excess in Wilson's disease is not affected by the administration of BAL or penicillamine.

Varying combinations of these therapeutic measures have been found beneficial in management of patients with this disease. The optimum program of administration of the chelating agents has not been clearly defined. The natural course of the disease, though more often inexorably downhill if untreated, may be prolonged and subject even to periods of improvement. This makes evaluation of therapy difficult. Dramatic improvement, sometimes sustained for long periods, may follow the use of penicillamine or BAL.

## SUMMARY

1 Wilson's disease (hepatolenticular degeneration) is a rare inherited disease which usually occurs in young people. Pathologically it is characterized by cirrhosis of the liver and degenerative changes in the brain particularly in the basal ganglia. Kayser Fleischer corneal rings are pathognomonic of the disease. An increased absorption of dietary copper from the intestinal tract with consequent increased deposition of copper in the tissues can be demonstrated. The increased copper in the body is localized primarily in the liver, brain, kidneys and cornea. The administration of radioactive copper can increase the number of heterozygotes in whom abnormalities in ceruloplasmin can be detected.

2 Biochemical abnormalities in Wilson's disease are multiple. Low serum copper and decreased serum ceruloplasmin levels are observed in the majority of patients with the disease and are associated with an increased urinary excretion of copper. The plasma levels of amino acids and glucose are usually normal or slightly decreased. The serum phosphate level is often decreased and the serum uric acid usually decreased.

3 Abnormalities in renal function are demonstrable; they consist of a progressive failure of tubular transfer mechanisms for amino acids, glucose, uric acid and phosphate.

4 Family studies indicate an autosomal recessive inheritance pattern. Decreased serum copper and serum ceruloplasmin are occasionally found in clinically normal heterozygotes.

5 The pathogenesis of the disease is obscure. Whether it is a general abnormality of protein metabolism in which specific tissue proteins bind copper preferentially or whether as seems more likely the primary disturbance is one of copper metabolism remains to be determined. The abnormality usually results in a decreased synthesis of normal ceruloplasmin. However the possibility that synthesis of an abnormal ceruloplasmin may perhaps occur must be considered.

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## Chapter 26

### Hemochromatosis

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Hemochromatosis is a form of iron storage disease characterized by the deposition of abnormally large quantities of iron in various organs or tissues of the body. It was probably first reported as a morbid entity by Troisier in 1871 [1] under the designation *la cirrhose pigmentaire dans le diabete sucré* although Trousseau [2] probably described a case in 1865 in the course of a clinical lecture on glycosuria. The term *hemochromatosis* was first introduced by von Recklinghausen in 1889 [3] to describe the clinical triad of generalized pigmentation, enlarged and cirrhotic liver, and diabetes mellitus. He used the term because he considered the disease an example of a hemorrhagic diathesis. The excess pigment in the tissue was presumed to arise from the breakdown of hemoglobin. The disease has also been referred to as bronzed diabetes, pigment cirrhosis, siderochromatosis, and primary congenital idiopathic or endogenous hemochromatosis.

The term *primary hemochromatosis* will be reserved here for the idiopathic form of iron storage disease which in untreated patients eventually progresses to the classical pathologic triad of cirrhosis of the liver, brown discoloration of the skin, and diabetes mellitus. Primary hemochromatosis probably represents an inherited metabolic disorder as first suggested by Sheldon in 1927 [4]. The disease is now known to be associated with an increased gastrointestinal absorption of iron.

#### DEFINITIONS

It is necessary to differentiate among the various conditions associated with increased body iron stores (Table 26-1).

Hemochromatosis, the form of tissue iron deposition associated with characteristic morbid changes, may be primary or secondary.

*Primary or idiopathic hemochromatosis* is a specific disease entity



It is one of several forms of iron storage disease known collectively as hemosiderosis (a descriptive histologic term). The increased iron stores in primary hemochromatosis result from increased absorption of the mineral from a normal diet. The increased iron absorption is not secondary to any known cause such as increased oral iron intake or erythroid hyperplasia. It is believed that this disease represents a genetically determined error of metabolism. Primary hemochromatosis usually begins in adult life and is much more common in males than in females.

TABLE 461 IRON STORAGE DISEASE

| Type                      | Cause of increased iron stores                             | Percentage of diet iron absorbed |
|---------------------------|--|----------------------------------|
| Hemochromatosis           |  |                                  |
| Primary                   | Inborn error of metabolism                                 | Increased                        |
| Secondary                 | Increased erythroid activity<br>Excessive oral iron intake | Increased<br>Normal              |
| Siderosis                 |  |                                  |
| Localized                 | Pulmonary hemosiderosis                                    | Normal                           |
| Generalized               |  |                                  |
| Transfusion hemosiderosis | Transfused blood   | Normal                           |
| Cytosiderosis (Bantu)     | Dietary deficiency plus excessive dietary iron             | Normal                           |
| Kashin-Bek disease        | Increased iron content of drinking water                   | Normal                           |

Secondary hemochromatosis is a form of siderosis which may result from increased iron absorption secondary to known causes. The most common of these is erythroid hyperplasia associated with hemolytic [5] pernicious [6] primary hyperplastic refractory [7-9] or Cooley's anemia [10-12], and chronic erythremic myelosis [13]. The anemias are frequently megaloblastic [6-8, 14]. Hemochromatosis has also been documented in hereditary hypochromic anemias [15, 16]. Secondary hemochromatosis must be extremely rare in patients with aplastic anemia who do not have an erythroplastic marrow [17]. Patients who develop hemochromatosis in association with anemia have frequently received large amounts of transfused blood. It was once thought that the transfused red cells represented the sole source of the increased iron stores found at autopsy. Houston, however, reported a case in a man with long-standing macrocytic refractory anemia who had been given no transfusions [18]. Secondary hemochromatosis has been reported in patients with mild anemia who have received no transfusions but who have received large doses of medicinal iron for many years [1, 19]. One female patient was presumed to have taken over 25,000 gm iron over a 47-year period [20].

Secondary hemochromatosis is frequently indistinguishable pathologically from the idiopathic type [9 12 15 18]. The pathologic changes usually develop while the patient has a normal dietary iron intake. Increased iron absorption has also been demonstrated in this group of patients [31]. The secondary type differs from the primary in that it may begin in childhood [22] and there is equal sex incidence.

*Siderosis* is a condition characterized by increased tissue iron without many of the clinical and pathologic features of either primary or secondary hemochromatosis. The term was first used by Quincke [23] for accumulated iron deposits in the tissues and organs under various conditions. Localized accumulations of iron may occur as a result of hemorrhage into the tissues producing a siderosis localized to a particular tissue as in pulmonary hemosiderosis. Neumann [24] first proposed the term *hemosiderin* for the microscopically visible tissue iron containing brown pigment.

*Transfusion hemosiderosis* is due entirely to the overloading of the tissues from iron contained in transfused red cells and is thus a true hemosiderosis since the excess stores arise from hemoglobin iron. The first record of an example of this condition was made by Hark who described two cases of aplastic anemia [25]. Since that time the condition though rare has been reported in about seventy cases [26-33]. The quantities of iron deposited in the tissues usually do not equal those found in hemochromatosis except when 200 or more blood transfusions have been given. The iron is deposited predominantly in the reticuloendothelial rather than the parenchymal cells and is therefore especially heavy in the bone marrow kidney and spleen [33 34]. The serum iron may be normal. Finely granular cirrhosis of the liver cardiac dysfunction and fibrosis of the pancreas are not found diabetes mellitus gonadal hypoplasia and skin pigmentation occur infrequently and are usually mild. Increased iron absorption probably does not occur in patients not having erythroid hyperplasia but patients with this latter condition may have increased tissue iron stores secondary to transfused red cell iron and increased iron absorption.

*Cytosiderosis* is endemic in the Bantu in certain areas in South Africa and is probably not a specific disease entity. It is a pathologic process that appears to begin in childhood [35 36] and occurs in individuals with malnutrition or pellagra whose diet contains very large amounts of iron [37]. It may progress to fibrosis and siderosis of the liver but diabetes and fibrosis of the pancreas do not occur and cardiac function is not impaired [38]. Increased tissue iron may also occur in severe malnutrition [39].

*Kaschin Beck disease* is a form of iron storage disease that appears to be restricted largely to Manchurian hunters and farmers [40] and is endemic in areas where drinking water with a very high iron content (0.3 to 1.0 mg per liter) is consumed during childhood and puberty. The

major changes appear in the skeletal system as shortened long bones and short stature joint deformities and arthritis particularly of the interphalangeal joints. A minimal fibrosis of the liver may occur without cirrhosis pancreatic fibrosis, or cardiac dysfunction. Skin pigmentation and elevated serum iron values may be present.

## CLINICAL FEATURES

Hemochromatosis occurs sporadically throughout the world and although it is rare cases have been reported from most countries. There appears to be no particular geographic distribution but the incidence may be lower in areas where iron deficiency is prevalent and higher in areas where increased dietary iron intake or certain refractory anemias are more common [41-43]. Environmental and dietary factors other than iron intake may play a role in influencing the age of onset of the disease. The frequency of the disease has not been correlated with occupation or exposure to toxic agents.

Extensive descriptions of the clinical features of hemochromatosis have been presented by Sheldon [41], Hellmeyer [43] and several American authors [42, 44-46].

### *Symptoms and Signs*

The asymptomatic interval preceding the development of the clinical features covers many years and the disease is seldom recognized during this stage unless by chance as the result of a tissue biopsy or plasma iron determination. Although symptoms and signs may have their onset earlier the full complex has not been reported prior to the age of 20 years [41]. Hemochromatosis developing in early adulthood is frequently fulminating and carries an unfavorable prognosis. In most cases the symptoms start in the middle years (in 90 per cent after the age of 30). The disease starts earlier in males and is ten to twenty times more common in males than in females. The comparative rarity of hemochromatosis in females and the later age of onset may be due to the increased iron deficits that result from menstruation, pregnancy and lactation. Throughout the sexually productive years the average woman may lose from 10 to 15 gm iron through menstruation. A single pregnancy and lactation may contribute to the loss of another 0.5 gm iron.

The symptoms most frequently encountered initially are those related to the classical triad of diabetes, skin pigmentation and cirrhosis. Although the liver is the first tissue to contain large deposits of iron the early symptoms of weakness, malaise and weight loss may most likely be related to the diabetes mellitus. Skin pigmentation develops insidiously and is followed by symptoms related to cirrhosis of the liver e.g. abdominal pain and ascites. Dyspnea and edema resulting from impaired cardiac

function and loss of libido as a consequence of involvement of the endocrine organs (testes) may next make their appearance

Skin pigmentation and hepatomegaly are the most constant physical signs of hemochromatosis and develop earliest. The brownish pigmentation is primarily the result of increased melanin deposition in the skin and appears early, with the increased deposition of iron in the integument a brownish gray (metallic bronze or slate) color makes its appearance. Pigmentation of the skin is usually generalized but it appears first and is most intense over the exposed areas and extensor surfaces of the arms, external genitalia, nipples and scars. Except for the less frequent mucous membrane pigmentation the distribution and appearance of the brownish pigmentation resembles that seen in Addison's disease [47].

The liver usually becomes enlarged long before signs and symptoms of hepatic dysfunction develop. Fibrosis and dysfunction of the liver underlie such characteristic signs of cirrhosis as spider angiomas and palmar erythema. Body depilation, loss of libido, testicular atrophy and gynecomastia may also be partially related to liver failure. With the steady progression of fibrosis and liver failure, the signs and symptoms and liver function test results become indistinguishable from those of typical Laennec's portal cirrhosis. With the onset of portal hypertension splenomegaly may appear. Such complications as ascites, edema and bleeding from esophageal varices appear to be much less common in hemochromatosis with cirrhosis than in typical Laennec's cirrhosis. Many patients with hemochromatosis of long duration develop primary carcinoma of the liver [42]. Cardiac failure and primary carcinoma of the liver may frequently contribute to the ascites and edema.

X-ray examination of the abdomen may demonstrate an increased density of the liver because of the high iron content. This is sometimes not prominent as a sharp line just below the shadow of the right diaphragm [42].

The myocardium is gradually involved; this is manifested by the signs and symptoms of congestive failure and cardiac arrhythmias [48, 49]. When cardiac failure develops there usually is dilatation of both ventricles with a globular cardiac profile resembling that occurring with pericardial effusion. In the younger subjects cardiac disturbances may progress rapidly and lead to sudden death. Cardiac failure now constitutes the single leading cause of death in hemochromatosis prior to the use of insulin; the leading cause was diabetes. Hypertension is rarely seen in hemochromatosis.

Endocrine dysfunction is manifested by loss of body hair, testicular atrophy, gynecomastia and menstrual irregularities. Loss of libido progressing to complete impotency may develop. The effects may in part be the result of impaired liver function.

Pain in the extremities, cutaneous hyperesthesia and muscle and leg

cramps frequently appear as a late complication of hemochromatosis these symptoms do not seem to be attributable to the diabetes mellitus. Psychic disturbances may also appear, and iron may be deposited in various areas of the brain [50].

Iron deposits occur focally in the renal tubules but do not affect renal function. There is no significant hematologic disturbance in primary hemochromatosis and the bone marrow is usually normal. The absence of hematologic involvement usually differentiates the idiopathic or primary disease from secondary hemochromatosis.

### PATHOLOGY

The pathology of hemochromatosis has been frequently reviewed [28-32, 41, 42, 51].

The liver is usually greatly enlarged and has a rusty appearance with a 'hobnail' surface. Microscopically, there is an advanced finely granular cirrhosis of the multilobular or portal type. Areas of regeneration are prominent. There is degeneration of the liver cells and a large amount of the iron may be present extracellularly or within the Kupffer cells. The large size of the liver, the small regenerative nodules of liver cells and the absence of broad bands of atrophy characteristically differentiate this disease from postnecrotic cirrhosis. Hemosiderin and hemofuscin pigments are present and the former is strikingly increased within the liver parenchymal cells. In the various other forms of siderosis the iron content of the liver is usually not so great and the largest quantity of iron is within the Kupffer rather than the parenchymal cells [30, 33, 36]. In typical Laennec's cirrhosis with siderosis the iron is also predominantly within the Kupffer cells and the liver is usually small [31]. Although fibrosis of the liver may occur in other forms of siderosis, finely granular cirrhosis does not [30, 32, 36].

The pancreas shows a heavy brown discoloration and except for the liver contains the largest amount of iron. There is a great increase in fibrous tissue. The gland cells show degenerative changes and siderosis and in most cases the islet cells are affected. It has been proposed on the basis of studies of 62 patients with diabetes secondary to hemochromatosis that the diabetes does not predispose to renovascular disease and that the diabetes of hemochromatosis probably represents a true insulin deficiency [52]. Diabetes mellitus occurs less frequently in siderosis uncomplicated by secondary hemochromatosis [9, 33]. The salivary glands like the pancreas usually show a deep brown red discoloration and the glandular epithelium contains large deposits of hemosiderin.

The spleen is usually only slightly enlarged. Hemosiderin deposits appear in very much smaller amounts than in the liver and pancreas. This

■ in contrast to the greater splenomegaly and much higher iron content of the bone marrow kidney and spleen in transfusion hemosiderosis [33 34]

The mucous membrane of the entire gastrointestinal tract may be pigmented The hemosiderin deposits are heaviest in the glandular epithelium of the stomach duodenum and cecum although there appears to be no alteration in the structure of the organs

The heart is usually a deep brown color Microscopically the endocardium and pericardium are not involved and the pathologic changes are confined entirely to the myocardium which may contain large amounts of hemosiderin Degenerative changes are seen in the muscle fibers and the increased fibrous tissue may replace the muscle

The pituitary usually shows a brown discoloration from increased deposits of hemosiderin localized to the cells of the anterior lobe The testes are usually atrophic and on microscopic examination show atrophy of the germinal epithelium The deposits of hemosiderin are usually not large and fibrous tissue is not increased The ovaries usually do not contain increased quantities of hemosiderin The parathyroid glands are frequently heavily pigmented without overgrowth of fibrous tissue The thyroid also shows a brownish pigmentation and microscopically there are marked overgrowth of fibrous tissue and increased quantities of hemosiderin within the alveolar epithelium

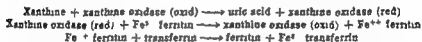
The adrenal glands do not appear altered grossly and there is no increase in connective tissue The microscopic changes are localized to the cortex and in most cases are confined to the outer zona glomerulosa Hemosiderin when present is always found in the glomerulosa and frequently only in this zone Hellier [47] reported a correlation between the degree of melanin pigmentation and the amount of iron deposited in the zona glomerulosa At autopsy one case of classical hemochromatosis with minimal pigmentation was found to have no iron deposits in the zona glomerulosa [38] In the various forms of siderosis the endocrine organs are not usually heavily laden with iron except for the deposits within the zona glomerulosa Some investigators [47 53-55] have alluded to a possible relationship between the skin pigmentation infrequent hypertension and more frequent hypotension and adrenal insufficiency secondary to the iron deposits in the adrenal cortex Studies of adrenal function in hemochromatosis have not been made although low values for both urine corticosteroid and 17 ketosteroid might be expected because of the cirrhosis [56] Simpson [37] reviewed the reported cases of Addison's disease with diabetes mellitus and could find only two with hemochromatosis Since the zona glomerulosa is most heavily laden with iron deposits it might be expected that aldosterone metabolism would be altered earliest

the apoferritin content of the mucosal cells of the stomach and duodenum [79] Ferritin is not normally present in the plasma but it may appear there in patients with toxic hepatitis [80]

Hemosiderin is the iron hydroxide-protein complex that is visible microscopically in the tissues as brownish granules. It remains insoluble under the conditions (neutral aqueous solution) used for the extraction of ferritin from the tissues [81] Hemosiderin contains relatively less protein than ferritin and consequently more iron. The iron content may range from 30 to 35 per cent and the iron to nitrogen ratio is 5-9:1. Hemosiderin and ferritin both give the Prussian blue reaction but in tissues the visible Prussian blue color is usually produced only by hemosiderin that is clumped in large granules. Studies with the electron microscope show that some of the hemosiderin deposits are contained within membranes ('siderosomes') and that some of the brown clusters of pigment may be predominantly ferritin [82]. Thus a significant portion of apparent hemosiderin may represent iron rich molecules of ferritin. Both hemosiderin and ferritin iron are utilized for hematopoiesis [83-84] although the latter is probably utilized more efficiently.

Transport iron in the plasma (3 to 4 mg) represents the link between the various tissue iron compartments. In the presence of oxygen ferrous iron entering the plasma is quickly oxidized to the ferric form which is then transported by a specific iron binding  $\beta$  globulin siderophyllin or transferrin [85]. The trivalent iron is bound to this protein with ionic bonds [86]. Normally the iron binding protein accounts for only about 3 per cent of the total plasma protein and is only about one third saturated with iron. Transferrin in man is not a single protein species. The different transferrins have been identified as the proteins involved in the genetically controlled variations of the serum  $\beta$  globulins in human beings [87]. A deficiency of transferrin has not been found in any disease. This protein has a molecular weight of 90 000 and can chelate a maximum of two iron atoms in the trivalent state (five-odd electrons). The transferrin forms a strong ionic bond with iron in neutral or alkaline solution to give a salmon pink color with an absorption maximum at 470 m $\mu$ . This property has made possible methods for its assay [88]. In vitro the reaction between iron and transferrin is reversible and is dependent on pH. The affinity constant at pH 7 is  $10^7$ . At physiologic pH the ionic iron concentration in the plasma is related to the level of bound Fe-transferrin and the concentration of unbound transferrin. Because of the high molecular weight of the Fe-transferrin it is unlikely that the complex normally leaves the vascular bed in more than trace amounts and thus the iron is presumed to pass in and out of the blood stream in ionized form [89] e.g. after oral iron feeding the plasma iron level increases without a change in the transferrin content. Normally transferrin has a biologic half life of approximately 12 days [90].

The biochemical mechanism for the release of iron from the tissue ferritin to the plasma transferrin has recently been investigated by Mazur and his coworkers. In rat liver slices *in vitro* [91] and in rabbits, guinea pigs, and dogs *in vivo* [92] the enzyme xanthine oxidase was shown to participate in the release of iron from liver ferritin. Tissue hypoxia was induced in all these experiments and was associated with an increase in purines in the liver tissue. Under anaerobic conditions and in the presence of ferritin the purines xanthine and hypoxanthine were oxidized to uric acid by the dehydrogenase activity of xanthine oxidase. In this reaction ferritin acts as an electron acceptor and its iron is reduced to the ferrous state. The ferrous iron can then be removed from the protein moiety by the plasma transferrin.



Thus the tissue oxygen tension levels of xanthine oxidase and xanthine oxidase substrates all may influence the regulation of the iron content of the circulating plasma.

Little is known of the mechanism for release of iron from the plasma transferrin for cellular utilization. On the basis of recent studies with the electron microscope it has been suggested that the immature red cells (erythroblasts) utilize a process called pinocytosis to obtain iron for hemoglobin synthesis by encompassing ferritin molecules directly from macrophages lying adjacent to the erythroblasts [93]. The importance of such a mechanism for incorporation of iron into marrow erythroblasts has not been established. It is known from *in vitro* studies that immature red cells are capable of taking up iron bound to the plasma protein transferrin [94-95]. Also there appears to be a very efficient system in the placenta for the transfer of plasma protein bound iron to the fetal circulation [96]. The reaction *in vivo* between ferric iron and protoporphyrin to form heme appears to be enzyme catalyzed [97]. In rat liver the enzyme is found in the mitochondria [98].

### *Iron Excretion*

The high molecular weight of transferrin and its great affinity for iron probably serve to protect iron from excretion. Normally less than 1 mg is excreted per day in the urine and feces [99]. As the result of menstrual loss the female may lose an average of about 1 mg additional iron per day. The concentration of iron in the bld is 1 to 2 mg per liter; however most of this is reabsorbed so that normally less than 0.5 mg is excreted each day [100]. The urine normally contains less than 0.5 mg iron per 24 hr [101]. In the presence of increased iron stores in man [102] and animals [103] iron excretion is usually slightly increased. It is decreased in states



of iron deficiency. In nephrosis large quantities of the iron transferrin complex may be excreted [104] and the urine may contain more than 15 mg iron per day [105].

### *Iron Absorption*

The normal adult accumulates his total body iron slowly during the first 20 years of life and thereafter remains in iron balance. The amount gaining access to the body by absorption equals the amount lost by excretion (0.5 to 1.5 mg per day). Alterations in excretion are minimal and it is generally agreed that the body content of iron is largely dependent on the amount absorbed. McCance and Widdowson [106] on the basis of their observations on the minimal excretion of iron from the body suggested that the mucosal cells of the gastrointestinal tract have an active role in the control of iron absorption. Hahn [107] from studies with radioactive iron found that iron absorption is not increased until 6 to 7 days after an acute hemorrhage; he suggested that the gastrointestinal mucosa does not increase iron absorption until the body iron stores have been depleted as a result of the accelerated hematopoiesis. He and others [108] found that the antecedent oral feeding of a large quantity of stable iron would block or inhibit the absorption of a dose of radioactive iron administered subsequently and proposed a 'mucosal block' to explain this observation. Granick [79] observed that feeding increasing amounts of iron increases the synthesis of apoferritin and results in the formation of increased amounts of ferritin in the intestinal mucosa. According to Granick, when only minimal amounts of iron are made available from the lumen of the gastrointestinal tract, iron reaches the blood directly through the mucosa. However, if much ferritin is present in the mucosa, it prevents further iron uptake. It was therefore postulated that the amount of iron absorbed depends on the relative state of saturation of the mucosal cells with iron and in turn is related to the level of tissue iron stores. Heilmeyer [109] and Wohler [110] failed to find evidence that the increased ferritin content of the intestinal mucosa reduces iron absorption. When the ferritin content of the mucosal epithelium was maximal, iron absorption continued and hemosiderin continued to increase in the interstitial tissues of the mucosa. If iron is given in excessive quantities [111], the mucosal cells of the intestine are unable to prevent its accumulation in excess of body needs. When more iron is present in the gut, more is absorbed, although the fraction absorbed may be smaller [112, 113].

The concept of the 'mucosal block' fails to explain the excessive absorption and heavy tissue iron deposits found in some subjects after the prolonged ingestion of large amounts of dietary [37, 38] or medicinal iron [5, 20]. Moreover, the diets of patients with primary and secondary hemochromatosis may contain a normal amount of iron, yet its absorp-

tion may be greater than normal [7, 8, 15 18 114-117] Also following experimentally induced hypochromic anemia in animals [118] and in normal subjects [63 64] iron absorption is increased After the anemia is corrected even though the iron stores remain depleted iron absorption reverts to normal In animals with experimentally induced anemia and increased iron stores iron absorption remains elevated [119 120] though it may be slightly depressed in animals when tissue iron stores are increased without an associated anemia [103 113 121] In the circumstances the ferritin content of the duodenum is elevated but the excess ferritin is deposited in the submucosa rather than in the mucosal cells [110]

The normal dietary intake of iron is 10 to 15 mg Less than 10 per cent of this quantity is absorbed through the gastrointestinal mucosa The main sites for absorption of iron appear to be the stomach and duodenum Other areas of the intestine absorb iron if it is present in a soluble form [122 123] Once absorbed the iron is transported via the portal vein blood to the liver In animals it has been shown that the intestinal lymphatic vessels do not transport the absorbed iron [124 125]

Both local intraluminal factors within the gastrointestinal tract and systemic or internal factors may influence iron absorption [126] With the use of  $\text{Fe}^{3+}$  salts it has been demonstrated that the normal adult absorbs 1 to 10 per cent of an oral dose of ferric iron salts if the dose is in the microgram to milligram range [127] With larger than milligram quantities the percentage absorbed decreases in proportion to the amount administered From 5 to 20 per cent of administered ferrous iron is absorbed Usually less than 10 per cent of food iron is absorbed [117 128 129] and the fraction absorbed varies with different foods Administration of a reducing agent such as ascorbic acid promotes the absorption of ferric salts but does not greatly promote absorption of food iron It has been shown experimentally that a low gastric pH will increase iron absorption by promoting the reduction of ferric iron and increasing the solubility of the iron salts Gastric acidity does not appear however to be necessary for absorption of the normal daily iron requirements Subjects with gastric achlorhydria do not show decreased absorption of iron and the addition of HCl to the feeding does not result in an increased absorption [128] The presence of phosphates or other substances capable of forming insoluble ferric or ferrous salts reduces the availability of iron for absorption [127 129 130] The presence of accessory factors that form soluble iron complexes (citrate) increases iron absorption Local secretions within the gastrointestinal tract may influence iron absorption e.g. ligation of the pancreatic ducts increases iron absorption in cats [131] and ethionine induced pancreatic damage is associated with a similar result in rats and mice [132]

The rate of erythropoiesis probably is the most important internal

factor influencing iron absorption [21, 113, 117] Patients with megaloblastic hemolytic and chronic iron deficiency anemias absorb increased quantities of iron, although in the first two groups iron stores are increased Iron absorption is increased in experimentally induced phenylhydrazine anemia in dogs [119] and rats [120] Following acute blood loss increased absorption is not noted for 5 to 7 days which is the time necessary for development of marrow erythroid hyperplasia [107, 113] In man altitude polycythemia will temporarily increase iron absorption [133] and hypoxia produces the same effect in rats [134] These physiologic states of lowered oxygen tension are associated with erythroid hyperplasia Patients with aplastic aregenerative anemias and marrow hypoplasia do not absorb increased amounts of iron, and do not develop siderosis unless they are transfused with red cells Transfusions of whole blood in sufficient quantities to depress marrow activity decrease iron absorption [113] In the absence of erythroid hyperplasia anemia per se and hypoxia do not increase iron absorption The anemia of infection is associated with decreased iron absorption at a time when the tissue iron may be normal or slightly increased [135] Pyridoxine deficiency in swine which is characterized by increased tissue and plasma iron levels and a hypochromic microcytic anemia with erythroid hyperplasia is associated with increased iron absorption [136] The level of the serum iron and the saturation of the plasma transferrin do not appear to influence iron absorption in dogs [137] or man [112] Decreased tissue iron stores in the absence of anemia do not promote [63, 64] increased absorption of iron In rats operative removal of the major quantity of storage iron by partial hepatectomy does not lead to increased iron absorption [133] In the rat increased storage iron produces a moderate decrease in iron absorption [121, 103, 113] or no decrease if anemia is present [120]

In the normal adult in addition to increased marrow activity pregnancy also may serve as a stimulus to increased iron absorption [139, 140] Infants and growing children also absorb increased quantities of iron [141-143] It seems likely that all these stimuli to increased iron absorption are mediated through a humoral agent Although some control of iron absorption seems necessary to restrict iron accumulation in the normal nonanemic adult the iron content of the tissues of the body does not appear to be related in any clear and direct manner to the regulation of iron absorption

#### *Rate of Turnover of Iron*

If it is assumed that the normal adult male has a total body iron content of 4 gm and that he is in iron balance it is possible to estimate the total body iron turnover by measuring quantitatively either iron absorption or excretion If approximately 1 mg iron is absorbed and excreted each day then approximately 9 per cent of the total 4 gm body iron (0.36 gm)

would be turned over per year, and its biologic half life would be about 77 years. Finch recently reported the results of a study on body iron turnover utilizing  $\text{Fe}^{55}$  and red cell sampling over a period of 3 to 5 years [144]. The calculated daily exchange of body iron (absorption or excretion) was 0.61, 0.64 and 1.22 mg in men, nonmenstruating women and menstruating women respectively. This represented a turnover of the total body iron miscible pool (red cell iron plus miscible tissue iron) of 8.3, 10.8 and 20.1 per cent per year. These data demonstrate that the miscible iron pool is appreciably smaller than the total body iron content. The miscible iron exclusive of the red cell iron pool probably does not include all the storage iron. Total body iron turnover studies in mice [103, 145] and ducks [146] have shown a more rapid turnover and the rate of turnover was proportional to body iron content.

After intravenous injection of  $\text{Fe}^{55}$  the plasma radioactivity follows a straight line on a semilog plot for a period of 6 to 8 hr in the normal subject [147]. When the curve is followed longer it deviates from linearity and may be described as a polynomial of at least two exponential terms [148-150]. The rate of turnover of the plasma iron pool may be calculated as the product of the total plasma iron and the fractional turnover rate derived from the initial slope of the curve. In the normal adult the turnover is 35 to 45 mg per day or roughly ten times the total plasma iron.



Fig. 26-1. Metabolic fate of iron. Normal daily rates of turnover through body compartments.

Assuming a mean red cell survival of 120 days and a synthesis of 0.8 to 0.9 per cent of the red cell mass per day, 20 to 25 mg of the total 35 to 45 mg of the rapidly exchangeable iron pool turned over per day is used for the synthesis of hemoglobin. The 10 to 15 mg portion of the labile (marrow) iron pool and the 3 to 4 mg from extracellular fluid, absorption and extramarrow stores are returned to the plasma (Fig. 26-1). This turnover may be increased in hemochromatosis more because of an increase in the total plasma iron than because of an increase in the fractional turnover rate.

The complexity of the  $\text{Fe}^{55}$  decay curve in plasma indicates that the plasma iron is miscible with several other compartments. Some of these are indicated in Fig. 26-1. From the  $\text{Fe}^{55}$  data it is possible to calculate the iron content of the labile iron pool, the quantity of iron that daily re-enters the plasma from this labile pool and the amount of iron leaving the labile pool each day for hemoglobin synthesis. From a knowledge of the iron content of hemoglobin it is possible to calculate the rate of hemoglobin synthesis.

## IRON METABOLISM IN HEMOCHROMATOSIS

In primary hemochromatosis, the quantity of iron in the diet is presumed to be normal. It would seem then that any explanation of the increased iron stores must assume an increased iron absorption at some stage of development of the disease. Quantitative measurements of iron absorption in hemochromatosis have yielded variable results [114-117, 129, 139, 141, 142]. This is probably partially attributable to the fact that iron balance studies utilizing radioactive iron yield variable results in normal subjects and because no strongly positive iron balance is needed to produce the increased iron stores so typical of this disease. The average patient with hemochromatosis has an excess store of 20 gm or more of iron at the time of diagnosis (30 or more years of age). This represents a positive balance of 2 to 4 mg per day over a period of 20 to 30 years or on a diet containing 10 to 20 mg iron per day an absorption of 20 to 30 per cent of the dietary iron rather than the normal 5 to 10 per cent. In a few patients the dietary history points to an increased iron intake secondary to the indiscriminate use of medicinal preparations of iron. These cases are more correctly classified as secondary hemochromatosis.

In a few studies of younger patients with hemochromatosis  $Fe^{59}$  balance techniques have demonstrated increased iron absorption [114-117, 141, 142]. In a recent study [117] in 5 patients an average of 10 per cent of an orally administered dose of radioiron added to a standard test meal was absorbed in contrast to an average of 5 per cent in normal persons. After phlebotomy the disparity between these two groups became more marked and up to 30 per cent absorption was found in patients with hemochromatosis at a time when the serum iron and iron stores were still high. This degree of absorption is more than is seen in patients with iron deficiency anemia or in normal subjects after phlebotomies.

Removal of injected  $Fe^{59}$  from the plasma of patients with hemochromatosis may be slow. Also the plasma  $Fe^{59}$  disappearance curve may show three distinct first-order processes rather than the two usually seen in normal subjects [140]. This suggests that significant amounts of the  $Fe^{59}$  reenter the plasma pool from at least two labile iron pool. Measurements of the slopes of the zero time intercepts permit a determination of the total quantity of iron removed via the marrow and storage each day: the quantity of iron in the labile marrow pool and other storage (liver) pool and the turnover of iron from the labile pool of the marrow used for hemoglobin synthesis. The plasma iron turnover is frequently increased [150, 152, 153] but not the rate of red cell synthesis. Red cell life span is normal. Since there is no increased synthesis of red cells in hemochromatosis increased erythroid activity a known stimulus to

increased iron absorption would not seem to be an etiologic factor in the pathogenesis of the disease

After the intravenous injection of  $\text{Fe}^{59}$  in vivo measurements over the liver show initially a greater than normal accumulation of iron in the liver. This continues for several days and demonstrates the great capacity of the liver to continue to accumulate iron in the presence of excess iron stores [154]

### *Iron Overload Studies in Animals*

Accumulations of iron have been produced in the tissues of experimental animals by various means. In no case has diabetes, cirrhosis, or myocardial damage followed [121, 155-162]. Accordingly there has been a reluctance to attribute the tissue damage in hemochromatosis to iron. However, experimental animals have usually not been fed dietary iron over a sufficiently long period of time to permit the accumulation of iron in amounts comparable to that seen in hemochromatosis. Furthermore, injected iron is distributed primarily in the reticuloendothelial system rather than in the parenchymal cells as in hemochromatosis. In both hemochromatosis and transfusion hemosiderosis (with secondary hemochromatosis) it seems necessary for the body iron content to reach 20 to 30 gm before the clinical picture of classical hemochromatosis makes its appearance. In man, if the bulk of the tissue iron is derived from transfused red cells, more than 50 gm iron may be present without any signs of severe tissue injury such as cirrhosis [163]. In the dog, after the administration of 25 to 35 gm iron intravenously, death results within 5 to 10 months, but no fibrous tissue reaction and no diabetes or cirrhosis is found [164].

## TREATMENT

The treatment of hemochromatosis should be directed at mobilization and removal of the excess tissue iron. Repeated phlebotomies are known to be effective in accomplishing these aims [63-65, 114, 165-173]. Patients with hemochromatosis withstand frequent phlebotomies (500 ml or more of whole blood each 4 to 6 days for many months) without developing more than a mild anemia. This implies that with this bleeding regimen the patients are able to increase their blood production four to sixfold above the normal rate. Failure to maintain a hematocrit above 35 with weekly phlebotomies suggests the presence of a complicating disease such as hepatoma or infection. Even in patients with a moderate degree of cirrhosis, it is usually not necessary to reinfuse the plasma.

In order to have an index of the extent of the iron stores, it is desirable to do a liver biopsy before starting the series of phlebotomies. Since each 1 ml of packed red cells contains approximately 1 mg iron, it is possible to remove only 10 to 15 gm iron per year, and thus in an average patient

the phlebotomies may have to be continued for 2 to 3 years. A gradual fall in the serum iron is usually the first sign of an approaching depletion of the iron stores when this occurs the interval between phlebotomies may be lengthened. A gradual decline in the hematocrit follows indicating a depletion of the iron stores. A repeat liver biopsy serves to confirm the presence of depleted iron stores.

It does not seem necessary to restrict the normal dietary intake of iron or to add phosphates to the diet since the amount of iron absorbed during the period of phlebotomies is small compared with that lost through the bleedings. Because these patients probably absorb an increased amount of the ingested food iron, it may be necessary to continue phlebotomies indefinitely at intervals of 3 to 6 weeks. The chelating agents presently available have no place in the therapy of hemochromatosis because they remove only trace quantities of tissue iron via the urine [174-175].

*Tissue injury (fibrosis of the liver and pancreas) and the clinical manifestations of hepatic, cardiac and pancreatic islet cell insufficiency, skin pigmentation and endocrine disturbances in patients with primary or secondary hemochromatosis strongly suggest a relationship between the iron content of the tissues and their injury. Reversal of many of the symptoms and signs and the lack of progression of the clinical picture of hemochromatosis in patients treated by phlebotomy also suggest that the pathologic features of hemochromatosis may be related to excess tissue iron. Since phlebotomy therapy has been in use for less than 10 years it is too early to predict whether it will materially alter the prognosis. However, experience to date demonstrates that the skin pigmentation decreases, cardiac function improves, liver dysfunction does not progress and the diabetes may be improved as judged by a reduction in insulin requirement. This form of therapy may not alleviate all the manifestations of the disease but it may well prevent further irreversible tissue damage.*

## GENETICS

Hemochromatosis occurs in both adult males and females although it usually occurs earlier in males. Thus it is not transmitted as a sex-linked defect. The disease appears sporadically. Following the initial case report of Frisch [176] on its occurrence in a family, numerous reports of familial cases appeared in the literature. Sheldon [41] evaluated the reported familial incidence in 14 cases but the data appeared reliable in only 5 of the families. Boulin [55] reviewed the data from a total of 27 cases with familial histories reported in 1953. The disease has been reported in 2 or more brothers in more than 25 different families [48, 176-190]. It has been observed in identical twin brothers on three occasions [180, 182, 183]. There are five reports of the disease occurring in successive

generations [42 182 185 186 191] There is one report of the disease occurring in a mother and her identical twin sons [182] and another report of the disease in both the mother and father, and a daughter [185] There are three case reports of the disease occurring as the result of consanguineous matings [186 190 191] In one such family appearance of a severe form of the disease in early adult life in three sisters and a brother [190] suggests the presence of a more severe juvenile form of the disease

The frequent occurrence of an elevated serum iron has been noted in the siblings or children of patients with hemochromatosis [42 186 192] In the study of Debré et al [186] elevated levels were not present in male or female offspring under age 15 nor in female offspring over 15 In males over 15 almost half showed elevated serum iron levels Also hyperpigmentation of the skin and diabetes have frequently been reported in relatives (children parents siblings) of patients with hemochromatosis Increased levels of serum iron and increased iron stores as measured by phlebotomy have been reported in the siblings of patients [42]

An analogy between elevated serum iron levels and hemochromatosis on the one hand and hyperuricemia and clinical gout on the other is perhaps instructive In both disorders the elevated serum concentration of the characteristic chemical substance is encountered much more frequently than the associated disease The onset of the chemical defect at puberty in males has been noted in both disorders Careful studies of inheritance of hyperuricemia have shown that it behaves as a dominant factor when viewed in successive generations but as a polymeric factor when viewed in sibling groups (Chap 21) One could equally well postulate that the genetic factors determining serum iron levels and hemochromatosis are polymeric On this basis one would expect elevated serum iron levels and hemochromatosis to occur in successive generations and in siblings and one would expect a severe and perhaps juvenile form of the disease in individuals inheriting it from both parents in offspring of consanguineous matings

Certain of the family data are also compatible with inheritance as an autosomal dominant trait with incomplete penetrance or even as an autosomal recessive characteristic However it appears unlikely to the author that the quantitative aspects of so complex a process as iron absorption would be determined by a single autosomal gene It is not known whether the genetically determined defect leading to increased iron absorption is in the mucosal cells or in some other body cellular enzyme system which in turn influences the absorptive capacity of the mucosal cells

Further studies of genetic factors in hemochromatosis are needed The combined application of studies of iron absorption and of serum iron



levels in families of patients with hemochromatosis offers a promising outlook for the acquisition of important information

## SUMMARY

1 Primary hemochromatosis is a form of iron storage disease characterized by excessive deposits of an iron hydroxide-protein complex called *hemosiderin* in various organs of the body. It is one of several forms of iron storage disease, and is not associated with known causes of excessive iron absorption such as erythroid hyperplasia.

2 Deposits of hemosiderin occur in liver, heart, the endocrine organs and elsewhere. The signs and symptoms of the disease arise from damage done these organs by the tissue reaction to the hemosiderin deposits. There is a curious metallic slate blue to brown pigmentation of the skin which is primarily due to melanin but also to deposition of hemosiderin. The pigmentation, hepatic cirrhosis and diabetes of the insulin-lack variety form the classical triad of the disease.

3 There is a small daily excessive absorption of iron in primary hemochromatosis. The cause for this is entirely unknown at present. There is no excessive excretion of iron. The net positive balance is only 2 to 4 mg per day. This excessive iron absorption is not associated with the common cause of increased iron absorption which is increased erythropoiesis.

4 There is no increase in intestinal ferritin in primary hemochromatosis nor is there any increase in plasma transferrin, the  $\beta$  globulin protein which normally transports iron. The plasma transferrin is more nearly saturated with  $\text{Fe}^{2+}$  than normally. The turnover of plasma iron is increased.

5 Treatment of the disease if begun early is helpful. It consists of systematic depletion of the iron stores by phlebotomy at appropriate intervals.

6 The disease probably has an autosomal recessive pattern of inheritance which is partially sex limited by virtue of the periodic loss of iron accompanying menstruation. Some families are reported in which the inheritance is more in keeping with a dominant form with incomplete penetrance. Hyperpigmentation of the skin, increased concentrations of serum iron and increased intestinal absorption of iron have been reported in otherwise normal relatives of patient with hemochromatosis indicating partial expression in the heterozygous state.

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## Chapter 27

### Periodic Paralysis

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*Jerome W. Conn and David H. I. Streaten*

Among the many conditions that may be associated with paralysis of the skeletal muscles very few exhibit the intermittency which is the characteristic par excellence of periodic paralysis. Repeated sudden bouts of flaccid muscular paralysis or weakness in persons otherwise enjoying apparently robust health constitute a striking clinical picture which is seldom likely to be confused with other conditions. The disorder has excited interest in the past 70 years out of proportion to its very low incidence because of the expectation that elucidation of its pathogenesis may contribute significantly to the understanding of muscle function in general.

Periodic paralysis may be defined as a disorder occurring usually in young persons characterized by attacks of weakness or paralysis that are spontaneous or in response to stimuli such as administration or endogenous release of salt retaining adrenocortical steroids. Such episodes are separated by periods of apparently normal muscular strength. Relaxation of the muscles predisposes to paralysis; muscular activity has a protective effect. When paralysis is severe it is associated with loss of tendon reflexes and loss of excitability of the muscles to electrical stimuli applied either through the motor nerves or to the muscles directly. Except in a few instances late in the disease there is no clinically evident muscular atrophy. No disturbance of sensation or of consciousness is present. The defect responsible for the condition is genetically transmitted in most cases but also appears to occur sporadically. The abnormality underlying periodic paralysis is now thought to be a disorder in the contractility of muscle resulting from electrolyte changes associated with and probably secondary to intermittent hyperaldosteronism.

#### HISTORICAL LANDMARKS

The literature prior to 1882 contains a few reports of patients who experienced repeated bouts of muscular paralysis which might well have

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disease could be associated with periodic paralysis. He demonstrated a greatly increased frequency of attacks during chronic administration of desiccated thyroid to patients with periodic paralysis even in the absence of any known abnormalities of the thyroid gland.

The electrolyte metabolic era was foreshadowed by the report of Biernard and Daniels [12] of a patient with familial periodic paralysis in whom the serum potassium concentration was found to be 13.38 mg per 100 ml (3.4 mEq per liter) during a mild attack and 17.87 mg per 100 ml (4.6 mEq per liter) when the attack had subsided. It was not until 1937, however, that Aitken and associates [13] established conclusively that both spontaneous attacks of periodic paralysis and attacks induced by administration of glucose and insulin were regularly associated with a fall in the level of serum potassium. This observation provided a rationale for the therapeutic use of large doses (12 gm) of potassium salts which they employed with great effect in several attacks. (Previously smaller doses had been given empirically by others [14-15].) Balance studies [16-18] demonstrated clearly that the fall in serum potassium level during attacks was not attributable to a negative potassium balance since urinary losses of this ion did not increase before attacks and fell sharply during them. Studies of the movements of potassium in and out of the extracellular fluid [19-20] suggested strongly that the fall in serum potassium level during attacks results from an excessive internal shift of potassium out of the extracellular fluid probably into the muscle.

The finding that deoxycorticosterone acetate and ACTH would precipitate severe paralytic attacks in patients with periodic paralysis gave rise to the suggestion that the adrenocortical steroids might be implicated in the pathogenesis of this disorder [21-22]. Studies of adrenocortical function in periodic paralysis by Conn et al. [23-24] have confirmed this suggestion by revealing a large increase in aldosterone excretion associated with sodium retention before attacks. The retention of sodium was thought to play an important role in inducing paralysis since patients could consistently be protected from attacks by depleting them of sodium and reducing their sodium intake to a minimum. The significance of these and other more recent observations [25] and their relationship to the observed potassium exchanges and electrolyte composition of skeletal muscle will be discussed in this chapter.

Within the past few years it has become evident that a group of patients previously considered to be suffering from periodic paralysis differ in certain clinical and biochemical respects from patients with the classical syndrome. These patients [26-43] now classified as having *adynamia episodica hereditaria* will be omitted from consideration in the account which follows and discussed in Chap. 28.

A number of excellent reviews of the literature on periodic paralysis

been attacks of what is now known as periodic paralysis. Paucity of the clinical data reported and the nature of some of the recorded observations make the evidence inconclusive, however. It is impossible to be certain for instance that the paralysis in some of these patients was not hysterical in origin [1] or that the disorder which in other patients was associated with malaria [2-4] was actually periodic paralysis.

The first report of a patient who indisputably had periodic paralysis was that of Shakhnovitch [5] in 1882. The patient was a man 44 years of age who had suffered from intermittent paraplegia for 25 years and whose father's death at 54 had been attributed to similar attacks of paralysis. Shakhnovitch also described for the first time the powerful build and splendid general health of the patient between attacks, the induction of attacks by cold, the almost invariable onset of attacks during sleep, the loss of tendon reflexes, and the lack of any disturbance of speech, sensory functions, or control of sphincters during the episodes of paralysis. His patient was considered to be suffering from a new form of neurosis for which he proposed the term *paraplegia spinalis intermittens nervosa*.

The observations of Westphal [6] in 1885 established that paralysis was associated with complete loss of muscular contractility in response to both direct and indirect stimulation with galvanic and faradic current.

In 1890 Goldflam [7] described the frequent occurrence of attacks on one day of the week (Friday in his case), the severe thirst which occurred during paralysis, and the profuse sweating which becomes obvious towards the end of the seizures. He drew attention to the tendency for the paralytic episodes to become less frequent with advancing years. He was also the first author [8] to observe vacuoles in microscopic sections of the skeletal muscles. Goldflam's theory that auto-intoxication is the cause of the malady was generally accepted for 30 years or more. The theory was based on the stubborn constipation experienced by these patients during attacks and on the toxic effect of organic constituents of their urine when injected into rabbits. Thus, while the clinical picture had been well delineated and the underlying histopathologic changes in the skeletal muscle had been described in great detail by the end of the nineteenth century, little progress had been made in tracing the pathogenesis of periodic paralysis.

Between 1900 and 1937 the main contribution to an understanding of periodic paralysis was the introduction of the experimental approach to the disease. This followed the discovery by Kramer [9] that the precipitation of attacks by the ingestion of large meals was due to their high content of carbohydrate. This observation was independently confirmed by Shinosaki [10] who was the first to use administration of glucose for the experimental induction of paralytic attacks. In company with other authors, Shinosaki also confirmed Kitamura's [11] report that Graves

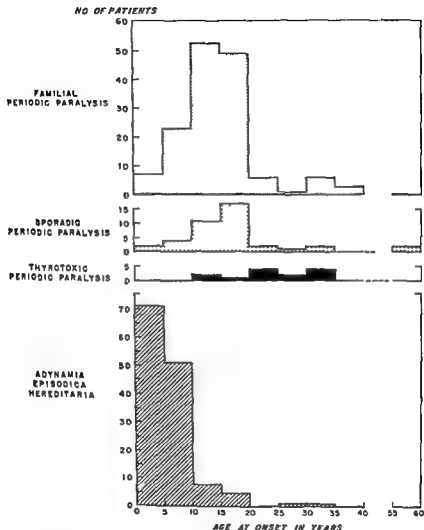


Fig. 26.1 Age of onset of attacks in representative series of familial periodic and thyrotoxic periodic paralysis and in adynamia episodica hereditaria.

to be more severe and more apt to end fatally in males than in females [48 56 62 63].

**Geographic Distribution and Racial Incidence** Although the disease is rare it has been reported from many parts of the world. Most of the patients have been Caucasians from Europe and North America. There have been a number of reports from Russia, Japan, India, and Brazil. No cases have been described among the Chinese or the Negro people.

have been published. Among the best are those of Goldflam [7, 44], Taylor [45], Schmidt [46], Janota and Weber [47], Dalinghaus [48] and Talbott [49].

## CLINICAL ASPECTS

### *Incidence*

**Age.** The age of onset of paralytic episodes has a wide range of variation, the extremes being 1 to 2 years [50] and 56 years of age [9, 51]. Attacks frequently begin during infancy in patients belonging to the group of *adynamia episodica hereditaria* (see Chap. 28) and during the third or fourth decades of life in patients with thyrotoxicosis [52-55]. After excluding these two groups from consideration, it appears that attacks have begun between the ages of 7 and 21 years in 93 of 100 patients described in the literature. These features are shown in Fig. 27.1 which gives the distribution of the ages at which paralytic episodes first occurred in the published reports of periodic paralysis (familial, sporadic and thyrotoxic) and of *adynamia episodica hereditaria*. The similarity in age of onset in familial and sporadic cases of periodic paralysis is in good accord with other evidence that the two conditions are identical except for the evident lack of hereditary transmission in the sporadic group. The striking differences between periodic paralysis and *adynamia episodica hereditaria* on the other hand support the view that these are different entities.

Several reports show a striking constancy in the age of onset of paralysis among most members of an affected family, e.g. 7 to 8 years in the family of Biemond and Daniels [12], 12 to 16 years in one of Mankowsky's families [56] and 17 to 19 years in MacLachlan's [57] and Khan's [58] families.

The severity and frequency of attacks usually increased to a maximum at about 20 years of age, remained substantially unchanged for 10 years or more, and became less severe and less frequent, especially in females, from about the age of 30 to 40 years, frequently disappearing some time between the ages of 32 and 73 years [7, 13, 17, 46, 56, 59]. A number of patients did not follow this usual benign course. Some persons continued to experience severe attacks in middle life or old age [7]. In a few of the larger families progressive muscular atrophy with permanent weakness or paralysis supervened in a number of the elderly patients [12, 60, 61].

**Sex.** In most families with periodic paralysis the disorder occurred more frequently among males than among females. The preponderance of males was slight in some families (Table 27.1a) and overwhelming in others (Table 27.1b). Among patients with thyrotoxicosis, males have been exclusively affected (Table 27.1c). The vast majority of sporadic cases have also been males.

Both in familial and in sporadic cases attacks of paralysis have tended

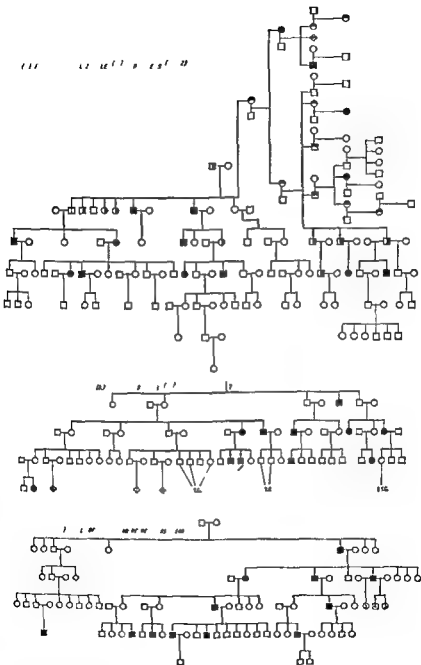


Fig. 2. Genealogy of periodic paralysis in three families (See Fig. 1 for explanation of symbol.)



TABLE 27.1 SEX INCIDENCE OF OVERT PERIODIC PARALYSIS IN A FEW OF THE REPORTED FAMILIES

| Type of case   | No. of cases |         | Reference |
|--|--------------|---------|-----------|
|  | Males        | Females |           |
| a Familial euthyroid cases with no marked male preponderance | 8            | 6       | [10]      |
|  | 6            | 5       | [44]      |
|  | 6            | 5       | [4]       |
| b Familial euthyroid cases with marked male preponderance    | 11           | 1       | [10]      |
|  | 11           | 3       | [50]      |
|  | 5            | 0       | [47]      |
|  | 8            | 0       | [8]       |
|  | 8            | 1       | [1]       |
| c Latents with thyrotoxicosis                                | 1            | 0       | [22]      |
|  | 4            | 0       | [53]      |
|  | 1            | 0       | [54]      |
|  | 1            | 0       | [118]     |
|  | 6            | 0       | [87]      |
|  | 1            | 0       | [55]      |
|  | 3            | 0       | [94]      |

and Dalinghaus [48] has drawn attention to the absence of the condition in the tropics

#### Condition between Attacks

Between attacks most patients have been free of symptoms and have had no abnormal physical findings. The one striking feature that many authors have commented upon has been the robust physique and the appearance of unusually good muscular development in most of the males [5, 9, 17, 46, 47, 64-74]. Even those of asthenic habitus or short stature [70] usually had an athletic build. The muscular size has at times suggested pseudohypertrophic muscular dystrophy, and muscular strength was sometimes less than normal [46]. More frequently, however, the strength of the muscles was as much increased as their size, so that the patients were good athletes who enjoyed a rugged outdoor life [47, 61, 75].

It has been reported that Chvostek's sign was present between attacks [76, 77] and disappeared during attacks in three patients [77] and that it remained positive in a fourth [76]. Goldflam [7] found reduced electrical excitability of the muscles between attacks in two patients, but Janota and Weber [47] considered that the electrical excitability of the muscles to direct and indirect stimuli was slightly increased between attacks.

#### Associated Disorders

In some patients the clinical features of thyrotoxicosis have been present, with enlargement of the thyroid gland and laboratory evidence

due to the high carbohydrate content of such a meal [9] administration of glucose has been used as an experimental method of precipitating attacks [10] Insulin alone will induce attacks [87] and the administration of both glucose and insulin is a very effective method of precipitating attacks [13 16 21 23 24 59 86 89 93-96]

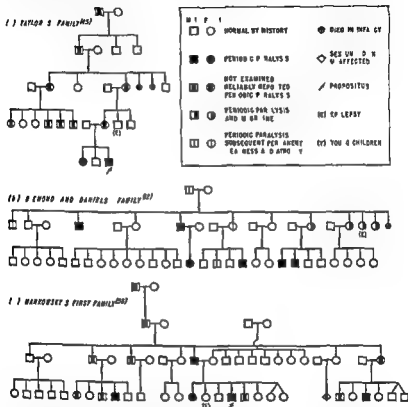


Fig 27.3 Genealogy of periodic paralysis in three families

**Rest and Muscular Relaxation** Rest especially following vigorous exertion appears to play an important role in precipitating attacks. Thus many writers [5 7 9 10 12 14-16 18 45-47 49 52 53 57-60 62 64 65 68 70-73 78 80 82 85 88 90 92 94 96-110] have mentioned that the severe attacks usually occur at night in the early hours of the morning or when the subject is at rest during the day. Conversely patients frequently learn that they can abort mild attacks (walk them off) by exercise [15 18 41 44 53 59 70 100 102]. That rest may have local effects on the muscles of these patients has been shown by the observation that paralysis may supervene in an immobile limb while active limbs escape [44 70 75]. The strong tendency for attacks to recur on the same

of hyperthyroidism (elevated basal metabolic rate (BMR), increased uptake of radioactive iodine by the thyroid and raised serum protein bound iodine (PBI) concentration) In these persons paralytic episodes started after the onset of thyrotoxicosis Shinosaki [10] and other Japanese authors have emphasized that the thyroid is frequently enlarged without evidence of hyperfunction of the gland but these observations were made before the advent of modern methods of appraisal of thyroid function

Various *neurologic disorders* may occur together with periodic paralysis *Migraine* is the most important of these A history of migraine in patients with periodic paralysis and in their relatives has been described frequently [12, 15, 47, 56, 57, 59-61, 68, 70, 71, 73-80 cf also 81] Migrainous headaches and paralytic attacks may occur in the same patient at different times or simultaneously and the occurrence of a headache has even seemed to protect some patients temporarily from an attack of paralysis acting as a type of 'equivalent' for attacks of periodic paralysis [52, 60] Gardner [63] MacLachlan [57] and Holtzapple [60] have all described the occurrence of headaches for several years followed in the same patient by the onset of paralytic attacks and cessation of the headaches Of the 14 patients with periodic paralysis in Holtzapple's family [60] the disease was transmitted through a parent with paralytic attacks in 10 instances and through a parent who had frequent sick headaches in the remaining 7 instances (Fig. 27.2a) If migraine and periodic paralysis constitute different modes of expression of the same abnormal gene as some of the evidence might suggest (see Genetics further on) then the incompleteness of penetrance in this family—and possibly in some of the others in the literature—would be more apparent than real *Epileptic seizures* have been reported frequently in patients with periodic paralysis [5, 56, 72, 82] and in some of their unaffected relatives (Fig. 27.3) *Progressive muscular atrophy* on the other hand apparently develops mainly in patients who have experienced paralytic episodes for many years and not in unaffected relatives [12, 59-61] (Figs. 27.2a and 27.3b)

### *Factors Which Induce Attacks*

Four groups of stimuli are known to precipitate attacks of paralysis in patients with the disease

**"Stresses" of a Wide Variety** These have included infectious diseases [6, 57, 60, 83] surgical and accidental trauma [62, 83, 84] emotional excitement anxiety or anger [10, 15, 16, 22, 45, 59, 70, 85] severe muscular exertion [10, 45, 68, 78, 80, 86, 87] menstruation [15, 88] and exposure to cold [5, 46, 47, 57-59, 89-91]

**Increased Utilization of Carbohydrate** That the ingestion of a heavy meal particularly in the evening tends to precipitate an attack has long been recognized [7, 15, 16, 58, 59, 65, 68, 71, 72, 91, 92] Drinking beer has also been incriminated [63, 86, 91] Since the effects of a heavy meal are

overcome by actively exercising the affected muscles [5 7 10 16 41 53 59 70 100 102]

The muscles may be painful during attacks [90] but the pain may often be relieved by a change of position [52] Several authors have been struck by the swelling of the affected muscles which gave them a firm rubbery consistency during attacks [5 57 59 69 71 83 85 89 105] Measurement of the affected limbs has shown that the apparent swelling has frequently been associated with an increase in circumference during paralysis [69 72 90 110 113] Contraction of the affected muscles on direct or indirect stimulation with galvanic or faradic current is reduced or completely abolished in proportion to the severity of the paralysis [4 6 7 13-15 68, 87 106] The deep tendon reflexes are reduced or abolished but the abdominal and cremasteric reflexes may be unaffected There is no loss of any sensory modality and no impairment of mental faculties or consciousness during even the severest of attacks

The myocardium may be affected along with the skeletal muscles during paralytic seizures Oppenheim's [106] original description of transient bradycardia and cardiac dilatation with the appearance of a systolic murmur at the apex believed to result from mitral insufficiency during attacks has been confirmed in part or entirely by many observers The bradycardia which is typical of the fully developed attack [68 90 95 106] is preceded by tachycardia before and early in the attack [14 46 47] The clinical evidence of transient cardiomegaly [10 46 66 68 106] has been verified by x ray [21 77 113] Elevation of blood pressure [10 46 66 110 114 116] and arrhythmias [10 44 60 103] have been reported during attacks

Gastrointestinal features of the attacks include occasional nausea and vomiting [46 66 77 78 95 102] and constipation bowel movements occurring seldom if ever during severe attacks [7 12 46 66 71 80 100 116] The possibility that these findings might result from loss of normal intestinal peristaltic motility was suggested to Dalinghaus [48] by the reports that bowel sounds are absent during attacks [46 80]

Changes in the intake and urinary output of water or both have frequently been described Thus severe thirst and increased imbibition of fluids may occur before or early in attacks [7 10 12 16 47 72 106 116] In contrast whether fluid intake is increased or decreased urine is seldom passed spontaneously during an attack [12 14 45-47 66 69 71 74 77 78 89 90 100] When the urine volume has been measured in relation to the fluid intake positive water balance has been found during paralysis [49] Severe sweating may occur during paralytic seizures and usually tends to usher in the phase of recovery [6 7 10 12 14 46 47 57 63-72 84 90 100 106]

**Phase of Recovery** The commencement of improvement is frequently evident clinically by the occurrence of a bout of severe sweating and by

day of the week, usually on Sundays or Mondays [7 10, 15, 65 68 73, 90] may be related to indulgence in more relaxation and a greater intake of food during the week end, or in some cases to increased athletic activity in the presleeping hours

**Drugs** Drugs which have been used to stimulate attacks include epinephrine [10 16, 59, 87, 95, 110-112] deionized thyroid [10 15 87] parathyroid extracts [10] adrenocortical extracts [90] deoxycorticosterone acetate [21 25] ACTH [22] 2 $\alpha$  methyl 9 $\alpha$  fluorohydrocortisone [24] and ammonium glycyrrhizinate [25]

### *Prodromal Symptoms*

While many patients are unaware of an impending attack until they awake paralyzed in the early hours of the morning some patients have experienced recognizable prodromata. The commonest appear to have been sensations arising from the muscles, described variously as aches and pains or cramps in the muscles [6 10, 14 16, 53 68 90, 100 109] stiffness or a tight feeling [15 57 71 86 93] and fatigue [9, 10 52 60 72 98]. Other patients have noticed numbness tingling and other paresthesias [6, 55 60 71 75 78] apprehension irritability or excitability [65 67 72], and an increase in thirst preceding attacks [9 16 45, 71, 72]

### *Paralytic Attacks*

Paralytic attacks may occur from once in a lifetime [47] to once a week [7 10, 15 65, 68 73 90] there may even be nightly attacks for 10 years [71]

Abortive attacks may last for less than an hour while attacks with complete paralysis usually last 6 to 24 hr. More severe seizures may endure for 1 or 2 days while Donath [84] and MacLachlan [57] have both recorded authentic instances of attacks lasting 8 days

**General Description of Attacks** In attacks which develop while the subject is awake weakness and then paralysis usually appear first in the lower limbs then in the arms trunk and neck in that order [6 7 14]. More characteristically patients are awakened in the early hours of the morning with widespread flaccid paralysis which renders them helpless

The diaphragm the muscles of the face and mouth and those involved in speech and swallowing are spared with rare exceptions such as in Putnam's [65] patient who in some of his severe seizures could move only his lips and eyes. On the rare occasions when death has ensued it has usually occurred during an attack involving the muscles of respiration [57] (see Mortality and Pathologic Findings further on in this chapter)

Many attacks are only partial involving a few muscle groups a single extremity or two limbs on the same side of the body or opposite sides—i.e., hemiplegia or paraplegia [90]. Minor attacks of localized weakness may occur during the day it is these attacks which can frequently be

sistent and is of doubtful significance. On two occasions endogenous creatinine clearance showed a slight reduction of questionable significance on the day of an attack [117-122].

*Urinary creatine* has been reported increased between attacks [95] to fall before the day of paralysis [21] and to rise during attacks [18-100].

*Urinary excretion of phosphorus* tends to be reduced in attacks roughly in parallel with urinary potassium [16]. An increased output of phosphorus after the attacks [16-123] is associated with no increase in the output of calcium and is therefore probably derived from muscle rather than from bone [123].

*Excretion of calcium and magnesium* has been found to be increased during attacks [10-18].

Changes in urinary excretion of sodium potassium chloride nitrogen uric acid pepsinogen 17 hydroxycorticoids 17 ketosteroids and aldosterone will be considered in detail in a subsequent section (see Metabolic Studies further on).

#### BIOCHEMICAL CHANGES IN BLOOD

The fall in serum potassium [12-13-16-21-23-49-55-59-74-94-96-98-119] and the rise in serum sodium concentration [23-24] will be discussed more fully later (see Metabolic Studies below).

Serum inorganic phosphorus falls for a few hours. This was reported first by Aitken et al [13] and was confirmed by many others [16-21-74-95-115]. The fall per se is probably of little or no importance in paralyzing the muscles since the infusion of sodium phosphate does not prevent paralysis [115].

Blood cholesterol concentration usually rises during attacks of paralysis [119a].

No change has been reported in blood pH [69], blood urea [13-103], serum calcium [10-13-18-21-80] or serum magnesium [13-18-21] although Yoshimura [124] found a rise in serum magnesium during attacks. Goss et al [103] reported that the cephalin flocculation and thymol turbidity tests, icteric index and serum total proteins were normal in their patient.

#### ELECTROMYOGRAPHIC AND CHEMICAL CHANGES IN MUSCLE

##### *Electromyographic Findings*

These have been described in detail in two patients with periodic paralysis by Lichler Jantz and Jung [96]. In the intervals between attacks the biphasic muscle action potentials were found to be normal in duration and shape. Rapid faradic stimulation however caused rhythmic fluctuations in the amplitude of the action potentials. These were more pronounced than the rhythmic changes seen in normal subjects indicating

the onset of diuresis [24, 46, 49 66] Muscles which were paralyzed first are usually the last to recover their function strength returning first to the neck and arms Recovery is usually complete in 1 to 3 hr from its commencement but it may take a day or more Pain stiffness or soreness in the muscles may persist for a day or two after the attack [60, 71 108] Patients customarily lose 1 to 3 kg in body weight because of the diuresis which occurs during recovery [117]

## LABORATORY FINDINGS

### ROUTINE LABORATORY FINDINGS

**Urinalysis** Transient mild proteinuria [9, 10 12, 46 47, 56] glycosuria [9 10] acetonuria [10 47, 118] and cylindruria [118] have been described during the attacks

**Hematologic Changes** Goldflam [8] reported a consistent neutrophil leukocytosis with eosinopenia during the attacks and lymphocytosis with eosinophilia after the attacks These findings have been confirmed by other authors [14 56, 66] Sugimoto [92] described the occurrence of lymphopenia during attacks of paralysis in a thyrotoxic patient

**Electrocardiogram** The ECG is normal between attacks and shows the changes characteristic of hypokalemia during episodes of paralysis viz flattened or inverted T waves particularly in the chest leads depression of RS-T segment and the occurrence of large U waves giving rise to the appearance of a prolonged Q T interval [19 21 24, 47 56 59 71 74 80 96 102, 108 109 119] Grob et al [19] have pointed out that the hypokalemic changes in the ECG are more severe in these patients than in normal persons at the same level of serum potassium

**Cerebrospinal Fluid** It may contain a slightly increased amount of protein [80 103] but shows no abnormality in pressure or cell count [49, 62 103] The potassium concentration in the cerebrospinal fluid is unchanged [95 103] or may be lowered [120] during attacks of paralysis

**Radiologic Abnormalities** These are restricted to the evidence of enlargement of the heart during the attacks [21 77 113] Cerny et al [114] have reported bony bridging of the sella turcica in four out of five affected members of a family with periodic paralysis

**Other Tests** Electroencephalography has shown no abnormalities [108] The BMR is elevated during paralysis contrary to the expectation in a paralyzed person [71 88 121] Gastric juice has been reported to contain little or no acid during attacks [15 48 77]

### BIOCHEMICAL CHANGES IN URINE

**Urinary excretion of creatinine** has been reported by Mitchell et al [16] to be low before attacks and high after attacks A slight fall in creatinine output [117] has been observed during attacks the finding was incon-

significantly different from the findings in normal subjects in whom KCl administration was consistently followed by increased uptake without subsequent loss of potassium from the forearm tissues. In most instances improvement in muscular strength following KCl administration lagged behind the rise in plasma potassium concentration but rapid improvement occurred when potassium loss from the tissues started i.e. in the second and third hours after KCl had been given. These findings together with the observation of increased potassium uptake by the tissues even in mild attacks associated with no fall in plasma potassium concentration suggested that abnormal uptake of potassium by the muscles is more important than the fall in plasma potassium in inducing muscular weakness. This suggestion is in good agreement with the conclusions of Fichler et al [96] based on electromyographic evidence. It receives further support from the observation that during recovery from attacks there was loss of potassium from the forearm which was hastened by increasing muscular activity as recovery progressed.

Zierler and Andres [20] made similar observations on the arteriovenous differences of potassium in two of the patients with familial periodic paralysis investigated by Grob et al [19]. These authors suggested that the nocturnal onset of spontaneous attacks and their usual spontaneous recovery later in the morning might be due to an exaggeration of the normal diurnal rhythm of potassium influx into the tissues at night and potassium efflux from the tissues during the day.

### *Muscle Function Studies*

The investigations of Grob et al [19] have shown that the muscle end plate became resistant to the depolarizing effect of intraarterially injected acetylcholine during bouts of weakness. Attacks were also accompanied by reduced propagation of muscle action potentials across the muscle following a single stimulus to the ulnar nerve. It was concluded that the decreased responsiveness of the muscles to nervous stimulation during attacks might be due to the development of resistance to the depolarizing action of acetylcholine normally released from the motor nerve endings. Since an increase in the ratio  $[K_{\text{transcellular}}]/[K_{\text{intracellular}}]$  ( $[K_{\text{out}}]/[K_{\text{in}}]$ ) causes hyperpolarization in experimental animals it was suggested that a similar change in this ratio might produce hyperpolarization of the motor end plate during attacks of periodic paralysis with resulting loss of muscular responsiveness to nervous stimulation. Similarly, hyperpolarization of the entire muscle membrane might bring about the observed reduction in the spread of the excitation wave to other parts of the muscle fiber.

The studies of Grob et al [19] confirmed the observation of Merton (reported by Mc Ardle [41]) that in patients with periodic paralysis the force of muscular contraction was reduced more rapidly and more severely than the amplitude or the propagation of the muscle action potentials.



that the muscle was abnormal even in the unparalyzed state. After the administration of glucose and insulin, but before paralysis supervened progressive widening of the action potentials occurred to two or three times their normal duration. Usually there was lengthening of the latent period between stimulus and response. As weakness advanced to complete paralysis there was progressive increase in the latent period, while the action potentials became more prolonged in duration and reduced in amplitude, until they could no longer be elicited shortly before all voluntary contractility of the muscle was lost. Rapid faradic stimuli induced a progressive increase in the rhythmic fluctuations in amplitude of action potentials until even before paralysis supervened the motor response to indirect faradization disappeared.

Eichler et al [96] have pointed out that the abnormalities in muscle action potentials started before paralysis of voluntary movement had taken place and before any other objective changes such as the fall in serum potassium level or alterations in ECG at a time when the only change was a subjective feeling of weakness. During the process of recovery from attacks the abnormalities in the electromyogram (EMG) were still present when the serum potassium had returned to normal or elevated levels and these abnormalities were restored to normal only after further delay. These findings indicate that the abnormalities in muscle action potentials do not result from the lowered level of serum potassium. Eichler et al suggested therefore that the EMG changes might reflect a movement of potassium into the muscle during paralysis.

#### *Potassium Exchanges between Plasma and Tissues*

Grob et al [19] have studied arteriovenous differences in plasma potassium concentration in three patients between and during attacks of weakness and paralysis. Their results indicated that the uptake of potassium from the arterial plasma by forearm tissues after administration of insulin or of epinephrine was significantly greater in the patients with periodic paralysis [19] than in the normal persons studied [19]. In 15 spontaneous attacks of weakness which followed evening meals high in carbohydrate content the patients showed significant increases in potassium uptake from the arterial blood into the tissues as compared with the potassium uptake by their tissues before the attacks. That the muscle and not the bone or skin was the site of the increased uptake of potassium during the attacks is suggested by the finding that the weakened contractions which could still be elicited during the attacks resulted in an increase in venous plasma potassium. This increase was four times as great as prior to the attack and twice as great as in normal persons following much stronger muscular contractions. The administration of KCl during attacks of paralysis caused initial uptake of potassium by the tissues followed paradoxically by a loss of potassium from the forearm.

TABLE 2<sup>2</sup> CONCENTRATIONS OF SODIUM AND POTASSIUM (IN MEQ/KG) IN SKELETAL MUSCLE IN PERIODIC PARALYSIS, HEALTHY PERSONS AND PRIMARY ALDOSTERONISM

| Initial | Diagnosis             | Date         | Paralysis | Muscle |        |        | Reference   |
|---------|-----------------------|--------------|-----------|--------|--------|--------|-------------|
|         |                       |              |           | Na     | K      | Na + K |             |
| RQ      | Periodic paralysis    | Oct 3 1936   | 0         | 46     | 75     | 121    |             |
| RQ      | Periodic paralysis    | Nov 29 1936  | 0         | 34     | 74     | 109    | 114.5       |
| RQ      | Periodic paralysis    | Oct. 31 1935 | 1+        | 48     | 82     | 130    |             |
| RQ      | Periodic paralysis    | Jan 14 1937  | 3+        | 5      | 85     | 97     | m           |
| RQ      | Periodic paralysis    | Jan 19 1937  | 4+        | 46     | 81     | 127    | 130.8       |
| WF      | Periodic paralysis    | Feb 8 1937   | 4+        | 47     | 8      | 129    |             |
|         | Hypothy               |              | 0         | 31     | 93     | 124    | m n [173]   |
|         | Hypothy               |              | 0         | 21     | 106    | 127    | 125.5 [174] |
|         | Hypothy               |              | 0         |        | 9      |        | [175]       |
|         | Hypothy               |              | 0         | 6.30   | 91.107 |        | [176]       |
| MW      | Primary aldosteronism | 1935         | 0         | 49     | 6      | 111    | [154]       |
|         | Primary aldosteronism | 1936         | 0         | 52     | 66     | 120    | [177]       |
| JS      | Primary aldosteronism | 1935-1939    | 0         | 35     | 69     | 104    | [117]       |
| AFE     | Primary aldosteronism | 1935-1949    | 0         | 54     | 59     | 113    | [117]       |
| GW      | Primary aldosteronism | 1935-1939    | 0         | 43     | 66     | 109    | [117]       |
| RMS     | Primary aldosteronism | 1935-1939    | 0         | 28     | 82     | 114    | 111.3 [117] |
| JH      | Primary aldosteronism | 1935-1939    | 0         | 31     | 83     | 114    | [117]       |
| RL      | Primary aldosteronism | 1935-1939    | 0         | 43     | 71     | 114    | [117]       |
| CG      | Primary aldosteronism | 1935-1939    | 0         | 39     | 81     | 90     | [117]       |
| MM      | Primary aldosteronism | 1935-1939    | 0         | 30     | 74     | 104    | [117]       |

After 44 days low sodium intake following total desalting with distilled water (10 mEq NaCl per day) (Tissot et al.)

Initial 4+ = mild hypokalemia 3+ = moderate hypokalemia 2+ = severe hypokalemia 1+ = mild hypokalemia

high sodium content of the muscles probably predisposed the subjects to paralysis since reduction of the sodium concentration by the desalting rendered the muscles immune to the paralytic effects of glucose and insulin and of mineralocorticoids (see under Sodium further on).

Paralyzed or weakened muscle consistently showed higher potassium concentrations (82-85-81-82 mEq per kg) than muscle sampled between attacks (75-74 mEq per kg). These results suggest that an acute shift of potassium may have taken place into muscle during the process of becoming paralyzed—a conclusion in good accord with the other evidence described above [19-21, 108]. In such paralyzed muscle the sodium concentration remains high and the potassium concentration is increased so that the concentration of (Na + K) becomes consistently higher

following nervous stimulation. For this reason it was postulated that the most important defect in periodic paralysis might lie in the contractility of the muscle itself rather than in transmission of the nervous impulse to the muscle. Again the hyperpolarization of the entire muscle fiber resulting from increase in  $[K^+]/[K^+]$  might be incriminated as an important defect in the muscular contractility, since actomyosin is kept in the uncontracted state by high membrane potentials [126].

### *Chemical Composition of Skeletal Muscle*

Evidence obtained by chemical analysis of muscle obtained by biopsy lends support to the more indirect evidence of potassium shifts in periodic paralysis described above. Jantz [21] reported that the concentration of potassium in muscle was increased at the height of an attack of paralysis to 710 mg per 100 ml (182 mEq per kg), and fell within half an hour of the recovery of normal muscle function to an approximately normal figure 360 mg per 100 ml (92 mEq per kg). Vastola and Bertrand [108] found that the onset of paralysis was associated with the movement of 120 mEq of potassium into each kilogram of muscle solids together with an increase of the water content especially in the extracellular fluid phase (i.e. the chloride space) of the muscle. The change in water content neutralized the effect of the potassium shift on the concentration of potassium in the sample of wet muscle which was unchanged by the occurrence of paralysis. Since the calculations of Vastola et al [108] show that the concentration of potassium in intracellular water actually fell from 196.5 to 190 mEq per liter when paralysis supervened their contention that the resting membrane potential had increased during paralysis is difficult to accept. However the increased quantity of potassium which these authors found within the muscle cells might be an even more important inhibitor of the association of actomyosin than an increased concentration of potassium [126].

More recent determinations of the electrolyte composition of muscle in periodic paralysis carried out in the authors laboratory (Table 27<sup>2</sup>) are in general agreement with the results of Jantz [21]. Between attacks of paralysis the concentration of potassium in the muscle was low (70 mEq per kg) probably indicating chronic potassium deficiency. The sodium concentration (46 mEq per kg) was considerably elevated as is usual in chronic potassium deficiency [127]. In fact the muscle composition between attacks closely resembled that seen in primary aldosteronism. "Desalting" the patient with diuretics reduced the sodium concentration in the muscle to 34 mEq per kg without materially affecting the potassium concentration. Since the sodium concentration in the muscle was high between attacks and was increased only slightly or not at all when paralysis was induced it seems likely that the supervention of paralysis did not involve a further shift of sodium into muscle. On the other hand a

TABLE 2. 2 CONCENTRATIONS OF SODIUM AND POTASSIUM (IN MEQ/KG) IN SKELETAL MUSCLE IN PERIODIC PARALYSIS, HEALTHY PERSONS AND PRIMARY ALDOSTERONISM

| Pat nt | Diag            | Dt          | Paraly | Muscl |        |        | Ref         |
|--------|-----------------|-------------|--------|-------|--------|--------|-------------|
|        |                 |             |        | Na    | K      | Na + K |             |
| RS     | P l p ly        | O t 3 19 6  | 0      | 46    | 75     | 121    | m           |
| RB     | I od r ly       | N 9 19 6    | 0      | 34    | 74     | 108    | 114 5       |
| RA     | P od p rlysis   | O t 11 1955 | 1+     | 48    | 8      | 130    |             |
| RA     | I d p ly        | J 14 19 6   | 3+     | 5     | 85     | 137    | m n         |
| RA     | P d p ly        | F 1 19 19 7 | 4+     | 46    | 81     | 127    | 130 8       |
| WF     | P od p ly       | F b 8 1957  | 4+     | 47    | 81     | 128    |             |
|        | H lthy          |             | 0      | 31    | 93     | 124    | m n [173]   |
|        | H lthy          |             | 0      | 21    | 100    | 121    | 125 5 [174] |
|        | H lthy          |             | 0      | 88    | 88     |        | [175]       |
|        | H lthy          |             | 0      | 26-36 | 91-107 |        | [176]       |
| MW     | Prim ry ldost m | 19 5        | 0      | 49    | 88     | 137    | [174]       |
|        | Prim y ld t m   | 1956        | 0      | 5     | 88     | 120    | [176]       |
| JB     | Prim ry ldost m | 1955-1959   | 0      | 35    | 89     | 124    | [177]       |
| AFE    | P ry ld te m m  | 19 5-19 9   | 0      | 34    | 69     | 113    | [177]       |
| AW     | P m ry ldost m  | 1953-19 9   | 0      | 43    | 66     | 109    | m n [177]   |
| RMB    | P m y ll te m   | 19 5 1959   | 0      | 32    | 83     | 114    | 111 3 [177] |
| JH     | P m ry ldost m  | 1955 19 9   | 0      | 31    | 83     | 114    | [177]       |
| BL     | Prim y ld te m  | 1955 1959   | 0      | 43    | 71     | 114    | [177]       |
| GO     | Prim ry ldost m | 1955-19 9   | 0      | 39    | 51     | 90     | [177]       |
| MM     | P m y ld te m   | 1955 1959   | 8      | 30    | 74     | 104    | [177]       |

Aft 64 dy lw d m tak fl w g t l d ft g th ta l m l (D m )  
d m pt m (Thi m m)

N t 4+ = mpt q d npl g 3+ = m p ly f th l m t 2+ = w ak m f m  
d f g 1+ = mld k e m f m l g

high sodium content of the muscles probably predisposed the subjects to paralysis since reduction of the sodium concentration by the desalting rendered the muscles immune to the paralytic effects of glucose and insulin and of mineralocorticoids (see under Sodium further on)

Paralyzed or weakened muscle consistently showed higher potassium concentrations (82-85-81-82 mEq per kg) than muscle sampled between attacks (75-74 mEq per kg). These results suggest that an acute shift of potassium may have taken place into muscle during the process of becoming paralyzed—a conclusion in good accord with the other evidence described above [19-21 108]. In such paralyzed muscle the sodium concentration remains high and the potassium concentration is increased so that the concentration of (Na + K) becomes consistently higher

(130 137, 127 129 mEq per kg) than in the unparalyzed state (121 108 mEq per kg) and higher than in normal subjects and patients with primary aldosteronism. Lales et al [120] have confirmed the presence of raised intracellular sodium and reduced intracellular potassium concentrations between attacks and the finding of a higher intracellular potassium concentration during an attack. As suggested by Conn et al [23, 24] it is possible that the excessively high  $Na + K$  content of the muscles consequent upon an acute inward shift of potassium is the cause of the paralytic attacks: since sodium and potassium have similar effects on the association of actomyosin [128] and since the actomyosin system is sensitive to the total amount of intracellular  $Na + K$  and not to their molar concentration [126].

#### EXCHANGEABLE SODIUM AND POTASSIUM (Table 27.3)

In four patients with periodic paralysis, Streeten and Conn [75] have found the exchangeable body sodium ( $Na$ ) to be high normal or elevated between attacks (46.6, 48.3, 51.3 and 51.4 mEq per kg) the normal range for  $Na$  at 24 hr in the same laboratory is  $38.5 \pm 2.9$  (s.d.) mEq per kg. In

TABLE 27.3 EXCHANGEABLE SODIUM AND POTASSIUM IN PERIODIC PARALYSIS

| Patient                                  | Diagnosis                                 | $Na$<br>mEq/kg | $K$<br>mEq/kg  | $Na/K$    |
|--|---|----------------|----------------|-----------|
| R.S.                                     | Periodic paralysis                        | 51.3           | 35.3           | 1.44      |
| W.F.                                     | Periodic paralysis                        | 48.3           | 41.5           | 1.20      |
| G.L.                                     | Periodic paralysis                        | 51.4           |                |           |
| B.H.                                     | Periodic paralysis<br>with thyrotoxicosis | 46.6           |                |           |
| 8 normal males<br>(Streeten et al [5])   |   | $38.5 \pm 2.9$ |                |           |
| 20 normal males<br>(Sagild et al [199])  |   |                | $49.3 \pm 6.4$ |           |
| 7 normal males<br>(Robinson et al [151]) |   |                |                | 0.74-0.94 |

24-hr values using  $Na^{22}$  and  $K^{42}$  separately

two of these patients exchangeable body potassium ( $K$ ) was determined and found to be 35.3 and 41.5 mEq per kg these values are slightly low for young males (normal mean  $49.3 \pm 6.4$  (s.d.) mEq per kg [129]). The high  $Na$  results may reflect the paucity of body fat in these lean athletic individuals but the slight lowering of  $K$  is all the more remarkable for the same reason. The patient studied by McArdle and Merton [130] had an exchangeable body potassium (43.4 mEq per kg) that was considered normal though it was well below the value obtained in their one normal control (51.3 mEq per kg). The ratio  $Na/K$  in the authors' patients (1.44 and 1.20 Table 27.3) was extraordinarily

high for lean muscular males normal values being 0.74 to 0.94 for males [131]. Such elevation of the ratio is seen also in primary aldosteronism in conditions associated with edema and in states of severe chronic debility and inanition. It has never been reported in apparently robust young males in the absence of periodic paralysis or primary aldosteronism.

### METABOLIC STUDIES

#### *Potassium*

**Serum Potassium Concentration** Between attacks the serum potassium concentration is normal in some patients but in others it may tend to be low. In one patient [70] for instance the fasting level of serum potassium was below 4.0 mEq per liter on 26 out of 28 days between attacks and was 3.3 mEq per liter or lower on 10 of these days. Repeatedly lowered serum potassium concentrations between attacks are evident also in the protocols of the patients reported by Talbott [49] and by Gass et al. [103]. A rapid and profound fall in serum potassium concentration, frequently to as low as 1.5 or 1.7 mEq per liter is one of the most consistent findings in attacks of periodic paralysis in patients with the familial sporadic or thyrotoxic forms of the disease [12 13 16 21 23 49 55 59 74 94-96 98 119]. The occurrence of hypokalemia during spontaneous and induced attacks is seen in Figs. 27-4 to 27-11. Several authors have drawn attention to the variability in the level of serum potassium at which paralysis occurs [16 17 74].

**Fate of the Disappearing Serum Potassium** In 1938 Allott and McArdle [16] and Pudenz et al. [90] independently showed by the balance technique that the fall in serum potassium could not be attributed to losses of potassium in the urine or stool. On the contrary urinary potassium excretion fell strikingly during the attacks as many observers have subsequently confirmed [17 18 21-23 49 103 119]. In the absence of an increased external loss of potassium the fall in serum potassium was attributed to an internal shift from the extracellular fluid into some other compartment presumably intracellular. This conclusion was supported by the electromyographic and muscle composition studies described above.

**Changes in Urinary Potassium** In attacks of paralysis induced by glucose (150 gm) and insulin (20 units) or by the potent synthetic mineralocorticoid 2 $\alpha$  methyl 9 $\alpha$  fluorohydrocortisone (1 mg p.o.) the urinary excretion of potassium declined simultaneously with the fall in serum potassium as shown in Fig. 27-4. In the spontaneous attacks of paralysis too the reduction in urinary potassium output accompanied but never preceded the fall in serum potassium concentration. Reduced urinary excretion of potassium is therefore probably entirely a secondary phenomenon consequent upon the fall in serum potassium which results from internal sequestration of potassium in the muscles or elsewhere. It

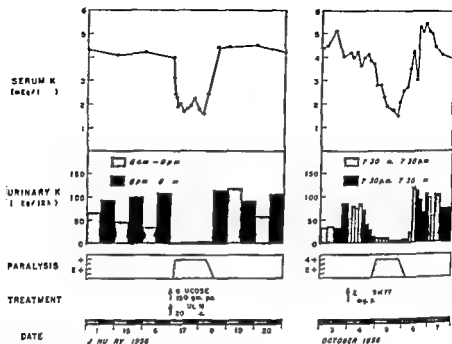


Fig 27-4 The time relationships between potassium changes in serum and urine during attacks of periodic paralysis induced by (1) glucose and insulin and (?) 2 $\alpha$  methyl 9 $\alpha$  fluorohydrocortisone

seems important that muscle function often begins to return before serum potassium values rise significantly. This phenomenon is preceded by sodium diuresis. After the end of the attacks diuresis of potassium usually occurred for 1 or 2 days.

### Sodium

**Sodium Retention** Severe attacks of paralysis either spontaneous or induced are usually preceded by sodium retention resulting in a positive balance of 100 mEq or more. Figure 27 5 shows the urinary output of sodium over a period of 8 weeks in a patient who experienced several spontaneous attacks of weakness and paralysis during this time. These included one spell of complete quadriplegia (designated as 4+ paralysis in the figure), three of severe paralysis of the limbs (3+ paralysis), one of weakness of the arms and legs (2+), and several of mild weakness of arms or legs (1+). Most of the severe attacks were preceded by sodium retention for 1 to 3 days. Sodium retention was greater and more consistent in the attacks induced by glucose and insulin (Fig 27 6) ■ methyl 9 $\alpha$  fluorohydrocortisone (Fig 27 7) DC\ ammonium glycyrrhizinate and ACTH [20] than in the spontaneous attacks. Unlike the reduction in potassium excretion which coincided with or followed the onset of

hypokalemia and paralysis the sodium retention preceded the paralytic spells and is therefore likely to be of greater significance in the pathogenesis of attacks [24]. A negative sodium balance usually occurred for 1 to 2 days after the end of the attacks and was sometimes of considerable magnitude. Sodium retention during attacks has been reported also by Pudenz et al [90] and Terrebee et al [18] and has been confirmed more recently by Eales et al [120] and de Graeff and Brocker [132]. An increase in exchangeable body sodium has been demonstrated to result from the sodium retention which preceded an attack of paralysis induced by 2 $\alpha$  methyl 9 $\alpha$  fluorohydrocortisone [24].

**Serum Sodium Concentration** The serum sodium tended to rise shortly before or during most seizures of severe paralysis or both in the three patients studied by Conn, Streeten et al [117]. This change was probably of little consequence in affecting muscle power but might have stimulated the thirst and oliguria which have frequently been commented on during attacks.

**Effects of Restriction of Sodium Intake** Remarkable protection from spontaneous and induced attacks was found to result from restriction of the daily intake of sodium to 10 mEq or less combined with initial desalting procedures (using diuretics such as chlorothiazide, mercurials and acetazoleamide) and quantitative replacement of the potassium losses resulting from the diuretics used [23-26]. Thus in contrast with the frequent spontaneous attacks on a normal sodium intake of 176 to 190 mEq per day shown in Fig. 27.5 there was only one bout of 3+ paralysis on the fourth day of a 23-day period on a 10 mEq sodium diet which was not combined with desalting. No attacks occurred in over 6 weeks on an 8 mEq sodium diet after initial administration of acetazoleamide and mercaptopimerin. During the latter period of 44 days two separate attempts to induce paralysis with 2-day courses of glucose and insulin administration and one attempt with 2 $\alpha$  methyl 9 $\alpha$  fluorohydrocortisone (see Fig. 4 in Conn et al [23]) were quite unsuccessful whereas previously and subsequently these stimuli never failed to induce paralysis in the same subject. Similar results [26] have been obtained in another patient who was readily paralyzed on the second day of receiving repeated doses of glucose (150 gm) and insulin (20 to 35 units) on a normal sodium intake (200 mEq per day). On an intake of 5 mEq per day however even without preliminary desalting this patient was protected from the paralytic effect of the same course of glucose and insulin administration. These observations on the protection afforded by a low sodium intake have been completely confirmed in two other patients studied by the authors including one with thyrotoxicosis [25] in the first of two studies by Eales et al [120] and in the less severe of two patients reported in brief by de Graeff and Brocker [132]. It is not clear whether the incomplete protection afforded by a low sodium diet in the other studies



of Lales and de Graeff et al might have been attributable to inadequate sodium depletion or to failure to replace the potassium lost by the action of the diuretics used

### *Chloride*

Changes in the urinary chloride excretion followed closely the changes in the urinary output of sodium. There was reduced urinary excretion before and during attacks and diuresis at the end of the attack [18, 23-29]. Like the serum sodium, the serum chloride concentration tended to rise before and during attacks, and this change was associated with approximately reciprocal changes in  $\text{CO}_2$  combining power [18, 25].

### *Water*

**Total Body Water** In many of the severe spontaneous and induced attacks observed in three patients on a constant diet [20] the sodium retention which usually preceded paralysis was associated with gain in weight signifying retention of water. An increase in body weight before attacks was observed previously by Neel [90] who attributed the gain in weight to the fall in urine volume which he and others [69] had observed. On the other hand, Ferrebee et al [18], Talbott [49] and Danowski et al [119] found negligible changes in body weight before attacks. The weight gain in the patient studied for the longest period of time by Conn et al [23-25] was greatest before the attacks which followed administration of the potent salt retaining substances DCA (3.2 kg) and ammonium glycyrrhizinate (2.2 kg). It was smaller before attacks which occurred after 2 $\alpha$  methyl 9 $\alpha$  fluorohydrocortisone (1 kg), glucose and insulin (1.1 kg and less than 0.5 kg) and spontaneously (0.3, 0.8, 0.2, -0.2, 0.1, -0.3, -0.5 kg). Poor correlation between the magnitude of weight change and the severity of the attacks suggested that an increase in total body water was of little or no importance in the genesis of the paralytic seizures. Support for this suggestion was derived from the finding that no paralysis resulted from the administration of pitressin tannate in oil despite an increase in the body weight of 3.2 kg in 3 days (see [20]). It may have been important that the severe retention of water induced by the pitressin tannate was associated with a negative sodium balance of 133 mEq (excluding fecal losses). This natriuresis was similar to that which follows the administration of pitressin to normal subjects, in whom it has been shown to be associated with reduction in aldosterone output [133-135]. There are a few reports of paralytic seizures which occurred after the administration of water [17, 136] but other authors [89, 103, 137] have failed to confirm that water loads even as large as 4-11 liters would precipitate an attack.

**Changes in Fluid "Compartments"** Ferrebee et al [18] and Danowski et al [119] found that paralytic attacks were associated with no signif-

icant change in extracellular fluid volume Danowski et al [119] observed a rise of 17 per cent in the plasma volume during attacks however Talbott [49] found plasma and extracellular fluid volumes to be increased in his patient as much as during attacks of paralysis In a single determination by Streeten and Conn [75] the bromide space between attacks was greater than normal Whether this implied an increased extracellular fluid volume or abnormal permeability to chloride of the tissues (muscle for example) which are usually relatively impermeable to this ion was not determined McArdle and Merton [130] found that the cellular water content was decreased to 21.4 liters in a patient with periodic paralysis between attacks compared with a normal value of 30.5 liters

Inadequacy of the available evidence makes it difficult to be certain of real changes in the internal distribution of body water in periodic paralysis although the published results suggest an expansion of plasma volume with conflicting evidence bearing on changes in the extracellular and intracellular compartments If such changes were aggravated before attacks one would expect them to result in a fall in the hematocrit In studies of three attacks it was found by Ferrebee et al [18] that the hematocrit remained unchanged early in the attacks but showed a downward trend during attacks from a mean value of 42.5 per cent at the onset to 39.7 per cent at the end of the attacks Since these changes did not precede the paralysis and since the downward trend in hematocrit during paralysis continued until 24 hr after complete recovery in the two patients in whom it was recorded these observations suggest that the development of paralysis was not associated with any significant internal shift of body water

### CHANGES IN ADRENOCORTICAL FUNCTION

#### *Steroid Excretion*

Apart from reports of occasional 17 ketosteroid values [108-114] no systematic studies of steroid excretion had been reported in patients with periodic paralysis prior to the work of Conn Streeten et al [20] The urinary excretion of 17 hydroxycorticoids [138] 17 ketosteroids [139] and aldosterone (estimated by bioassay as described by Streeten et al [140]) is correlated in Fig. 27.5 with the changes in serum potassium concentration and urinary sodium output in a number of spontaneous attacks observed in one patient In Fig. 27.6 a similar correlation is shown in four attacks induced by glucose and insulin in the same patient and in two others with sporadic periodic paralysis The aldosterone values shown in Fig. 27.6 were obtained using the chromatographic method of Neher and Wettstein [140a] as modified by Louis [122]

**Urinary 17 Hydroxycorticoids** A definite increase in the output of 17 hydroxycorticoids occurred on the first and second days of paralysis

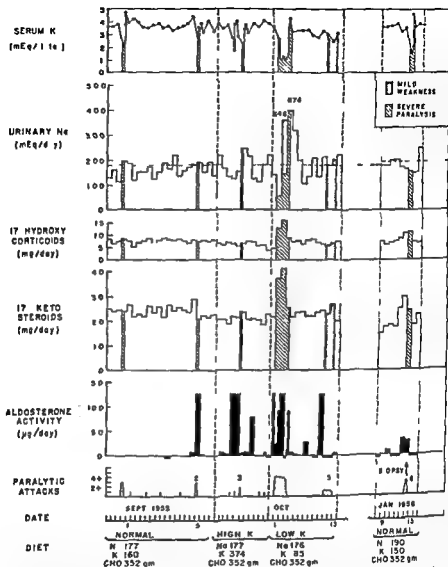


Fig. 27-5 Serum potassium and sodium and steroid excretion in patients with spontaneous attacks of periodic paralysis.

In this and subsequent figures illustrating metabolic data, severity of paralysis is indicated as follows: 4+ = complete quadriplegia; 3+ = severe paralysis of the limbs; 2+ = weakness of arms and legs; 1+ = mild weakness of arms or legs.

Electrolyte (e.g., sodium) intake is indicated by the dotted line, and urinary output is plotted from the base line upwards, so that when urinary output exceeds intake, the output column rises above the dotted line depicting intake.

The cross-hatched areas (Nos. 1 to 6) indicate the duration of major attacks of paralysis.

in the most severe spontaneous attack (Fig 27 5 No 4) and on the day before and the day of the next most severe spontaneous attack (No 6). An increase in 17 hydroxycorticoids was also seen in the two severe attacks (Fig 27 6 Nos 7 8) induced by glucose and insulin. The 17 hydroxycorticoid output was higher on the day of each of these attacks than on any other day of the study. The mild attacks, both spontaneous (Fig 27 5) and induced (Fig 27 6 No 9) were accompanied by no demonstrable change in 17 hydroxycorticoid excretion.

**Urinary 17 Ketosteroids** The 17 ketosteroids showed an undoubted increase in the two severe attacks [20] (Fig 27 5 Nos 4 6). Moreover even the less severe attacks were always associated with an increased output of 17 ketosteroids on the day of the attack (Nos 3 5) or the day immediately before (No 2) or after (No 1) the attack. Again the fact that the 17 ketosteroid output at the time of the attack was in every instance the highest excretion reported for 2 to 14 days before and after each attack suggests that these changes are significant despite the relatively wide day to day fluctuation in 17 ketosteroids in this patient. The increase in excretion of 17 ketosteroids *before* attacks (Nos 2 6) suggests that adrenal hyperactivity or whatever change in steroid metabolism caused this augmentation of 17 ketosteroid output might have been concerned in the causation and was certainly not the result of the paralytic attacks. This interpretation is strengthened by the observation that a severe attack of complete paralysis which occurred after the administration of the potent nonsteroidal sodium retainer ammonium glycyrrhizinate (a triterpene) was associated with no significant change in 17 ketosteroid output. Thus the 17 ketosteroids were 20.6, 18.9 and 18.1 mg before administration of the triterpene, 20.0 and 18.4 mg on the first and third days of its administration, 17.1 and 19.1 mg on the 2 days of the attack, and 17.0 mg on the following day. The evidence suggests therefore that the increased output of 17 ketosteroids before or during attacks of paralysis is a characteristic feature of the metabolic events leading to spontaneous seizures but one which plays no essential role per se in inducing paralysis. Cerny et al [114] in the protocol of one of their patients with familial periodic paralysis recorded an increase in the urinary 17 ketosteroids from 5.8 mg per day between attacks to 17.3 mg per day on the day of an attack. This observation is in agreement with those described above.

**Urinary Aldosterone Output.** The urinary excretion of aldosterone-like material was estimated by bioassay before, during and after five of the spontaneous attacks (Fig 27 5 Nos 2 to 6). In every instance a large increase in excretion of aldosterone-like substance occurred on the day before and usually also on the day or days of paralysis. Excretion of salt-retaining steroid was normal (less than 3  $\mu$ g per day) on several of the days between attacks but was not infrequently increased on two days

between attacks when a great increase in aldosterone output occurred sharp sodium retention also occurred (Sept 26 and Oct 7, 1955) In addition one of these days (Oct 7) was followed by the only reduction in serum potassium concentration below 4 mEq per liter observed among

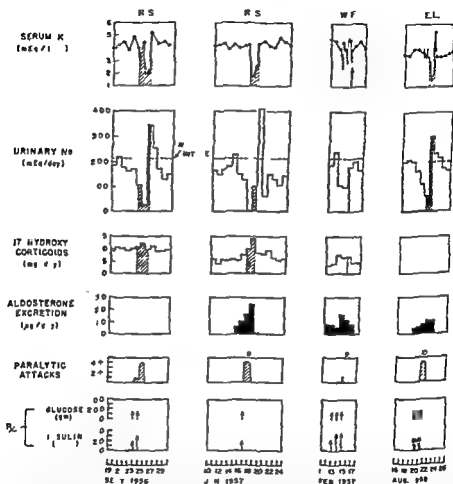


Fig 27-6 Serum potassium and urinary sodium and steroid excretion in attacks of periodic paralysis induced by glucose and insulin. Symbols as in the legend for Fig 27-5

52 determinations obtained between attacks in this patient. These findings may therefore have represented an 'abortive spontaneous attack.'

The attacks induced by glucose and insulin in the three patients (Fig 27-6) were also associated with increased urinary output of aldosterone (measured chromatographically in these studies). The more

modest rise in aldosterone output in these induced attacks might simply reflect the differences in technique or might indicate that the spontaneous attacks were really associated with far greater adrenocortical hypersecretion than occurred after glucose and insulin. Bearing in mind the obvious limitations of the bioassay [140-141] one is probably justified in attributing this difference to technical factors until further evidence to the contrary has been adduced. Eales [136] has confirmed the finding of an elevated aldosterone output during two spontaneous attacks.

Since the increased output of adrenocorticoids in the urine before and during attacks involved three different steroids and since paralytic attacks were associated with no obvious changes in hepatic or renal function it seems reasonable to conclude that increased adrenocortical secretion was the cause of the increased urinary excretion of these metabolites. This conclusion receives support from the evidence of the biologic effects of increased adrenocorticoid output.

#### *Biologic Effects of Increased Adrenocortical Steroid Output*

**Electrolyte Metabolism** Since the augmented output of aldosterone correlated reasonably well with the sodium retention and the decreases of serum potassium (Fig. 27-5) there is reason to believe that hypersecretion of aldosterone might have been the cause of these electrolyte effects. The rise in serum sodium associated with the attacks [23-24] was probably also attributable to hypersecretion of aldosterone. The urinary output of potassium was reduced (Fig. 27-4), rather than increased as would have been expected normally during greatly increased aldosterone secretion. Thus a fundamental defect in periodic paralysis (unavailability of potassium for urinary excretion during attacks) is again evident. It is significant that administration of mineralocorticoids to such patients results in the same abnormal pattern of renal excretion of electrolytes.

**Organic Metabolism** Changes in protein metabolism during paralytic attacks have been reported by Conn, Streeten, et al. [25]. Figure 27-7 shows the urinary nitrogen output before, during, and after 14 attacks of paralysis in one patient (R. S.). The duration of attacks is indicated by crosshatching. Fecal losses of nitrogen were not estimated except in the first attack (Oct. 1, 1950) in which the occurrence of a slightly negative nitrogen balance on the first and third days of the attack is shown. Urinary losses of nitrogen were so great on the day of or after each of the spontaneous attacks or both, however, that they frequently exceeded total nitrogen intake. The attacks induced by glucose and insulin (Nos. 10-11) and by 2 $\alpha$ -methyl-9 $\alpha$ -fluorohydrocortisone (No. 12) were also associated with a negative nitrogen balance, but no increase in urinary nitrogen output occurred in the attacks induced by DCA (No. 13) and ammonium glycyrrhizinate (No. 14). The steroid 2 $\alpha$ -methyl-9 $\alpha$ -fluoro-

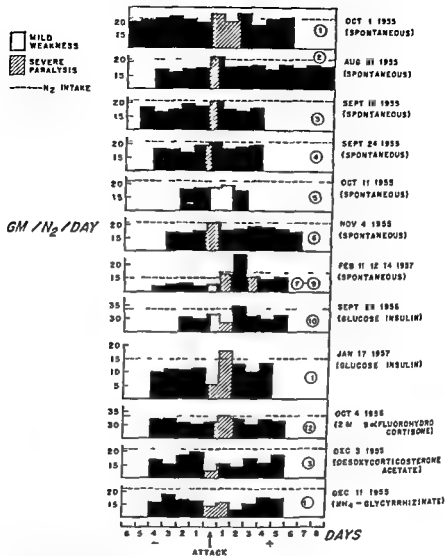


Fig 27.7 Changes in urinary nitrogen in a patient during 14 attacks of periodic paralysis. The data are plotted as in Fig 2.5

hydrocortisone has sufficient glucocorticoid potency to increase urinary nitrogen excretion when given to normal subjects in doses as low as 0.5 mg by mouth [142]. The increased nitrogen excretion observed in the patient with periodic paralysis given twice this dose (Fig 27.7 No 12) therefore might well have resulted from the glucocorticoid activity of the steroid administered and does not imply an increased endogenous release of glucocorticoids. DCA and ammonium glycyrrhizinate are both known to possess potent mineralocorticoid activity and to be practically

devoid of glucocorticoid activity. The absence of increased nitrogen excretion in the severe attacks which followed their administration therefore suggests that the excessive adrenocortical secretion of a glucocorticoid (probably hydrocortisone) in the spontaneous attacks neither played an essential role in the genesis of muscular paralysis nor resulted merely from the stress of the seizures.

Uric acid excretion was increased from a mean control level of 0.93 gm per day to 1.05 to 1.54 gm per day on at least one of the days in each of six spontaneous paralytic attacks studied in the same patient [25]. On the only day between attacks when the urinary uric acid excretion exceeded 1.05 gm per day there were also sodium retention, a fall in serum potassium to 3.9 mEq per liter, and increased 17-hydroxycorticoid excretion with increased aldosterone output on the preceding day, suggesting that an abortive attack was taking place. The increased uric acid excretion presumably reflected the effects of increased adrenocortical secretion of hydrocortisone.

**Other Effects of Adrenocortical Hyperactivity** Urinary ammonia was greatly increased on the day of paralysis in four spontaneous attacks and in one attack which occurred probably spontaneously on the second day of prednisolone therapy (following spontaneous increase in aldosterone secretion the day before the attack). The same phenomenon occurred during attacks induced by an upper respiratory infection and by administration of glucose and insulin. 2 $\alpha$ -methyl 9 $\alpha$ -fluorohydrocortisone (DCA) and ammonium glycyrrhizinate [25]. Increased urinary ammonia probably resulted from increased mineralocorticoid activity since it was seen in the attacks which followed administration of DCA and ammonium glycyrrhizinate and is known to occur in primary aldosteronism where there is no increase in 17-hydroxycorticoid excretion [122]. The resemblance of this increased urinary excretion of ammonia to that which occurs under the influence of excessive aldosterone secretion was further manifested by the striking phenomenon of its persistence through the second day of three severe attacks of paralysis when urinary pH was increased above 7.0.

*Urinary pepsinogen excretion* increased on the day of or after each of three spontaneous attacks: one attack induced by ACTH gel, one which followed an upper respiratory infection, two induced by glucose and insulin, and one precipitated by 2 $\alpha$ -methyl 9 $\alpha$ -fluorohydrocortisone [25]. A rise in the urinary excretion of pepsinogen may result from increased renal clearance of pepsinogen in response both to adrenocortical stimulation [143] and to infusion of hydrocortisone [144]. It has been encountered in primary aldosteronism [144a]. Increased urinary pepsinogen excretion in periodic paralysis therefore presumably results from the increased adrenocortical secretion of hydrocortisone or of aldosterone or both.



### SIGNIFICANCE OF THE INTERMITTENT ADRENOCORTICAL HYPERSECRETION

It has been shown that both spontaneous attacks and attacks induced by glucose and insulin are accompanied by a transiently increased secretion of adrenocortical steroids. This phenomenon, which frequently starts before paralysis supervenes, constitutes a characteristic feature of the paroxysms and is not merely the consequence of the "stress" of paralysis (see discussion under Organic Metabolism above).

#### *Elevation of Urinary 17-Hydroxycorticoids*

This probably results from increased secretion of hydrocortisone since the usual results of such an increase are readily recognizable: negative nitrogen balance [25] (see under Organic Metabolism above), greater excretion of uric acid [20], mildly elevated FBS [10, 20, 47, 56, 114], leukocytosis [8], lymphopenia [92] and eosinopenia [8]. These changes are quite mild, however. The administration of glucocorticoids in amounts equivalent to or even greater than the adrenocortical output of hydrocortisone which would be estimated to occur before or during these attacks (probably never more than twice the normal adrenocortical output of 25 to 35 mg hydrocortisone daily) does not cause paralysis [22]. For these reasons and because severe paralysis may result from procedures which do not increase glucocorticoid activity in the body (e.g. administration of DCA and ammonium glycyrrhizinate) there is little doubt that excessive secretion of hydrocortisone plays no essential role in the genesis of paralysis.

#### *Increased Output of Urinary 17-Ketosteroids*

This increase is more consistent than the augmentation of 17-hydroxycorticoid excretion. It may result from increased adrenocortical production of adrenal C<sub>19</sub> steroids, from increased hepatic formation of 11-oxygenated 17-ketosteroids from glucocorticoids, or from increased testicular secretion of androgens. In the absence of further evidence which might be obtained by chromatographic fractionation of the elevated urinary 17-ketosteroids, it is difficult to interpret the significance of this change. There can be little doubt that while this change too is an integral part of the metabolic events inherent in the genesis of paralysis it plays no direct role in paralyzing the muscles.

#### *Increased Output of Aldosterone*

This is the steroid change which most consistently precedes and accompanies spontaneous paralytic seizures. Furthermore, an increase in the salt-retaining, aldosterone-like activity in the body is the only known factor which is common to almost all, if not all, of the means by which

paralysis may be induced: emotional upsets [145] infections surgical and other physical trauma [146] increased rate of carbohydrate utilization resulting from glucose and insulin administration (see Urinary Aldosterone Output above) and the administration of ACTH [147 148] adrenocortical extract DCA  $2\alpha$  methyl  $9\alpha$  fluorohydrocortisone and ammonium glycyrrhizinate. There are therefore strong reasons for believing that a transient rapid increase in aldosterone production plays an important role in the pathogenesis of paralysis.

This evidence is strengthened by the observation in four patients [24 25] that the paralytic effects of glucose and insulin were completely abolished by feeding a low salt diet. Most if not all of the metabolic effects of aldosterone are known to be abolished by sodium restriction. This procedure may ameliorate or entirely reverse many of the abnormal features of severe primary aldosteronism [149]. Additional confirmation of the pathogenic role of transient hyperaldosteronism in precipitating attacks has been adduced by the administration of a spiro lactone (SC8100) to these patients. Adequate doses (500 mg q 6 h p o) of this drug which is known to antagonize the effects of liberated aldosterone [150 151 152] prevented the sodium retention which was otherwise always associated with the administration of glucose and insulin to one patient. In another patient with severe periodic paralysis associated with thyrotoxicosis the same spiro lactone protected the individual from paralysis during 3 days of glucose and insulin administration although 1 or 2 days of the latter treatment had previously paralyzed him consistently [25].

#### NATURE OF THE RELATIONSHIP BETWEEN TRANSIENT HYPERALDOSTERONISM AND PARALYSIS

Consideration of the evidence bearing on the following questions may serve to clarify the nature of the defect in periodic paralysis.

1 *Is the hyperaldosteronism due to an excessive stimulus to aldosterone production or to an excessive response by the adrenal gland to a normal stimulus?* Excessive secretion of aldosterone has been observed repeatedly in spontaneous attacks which were preceded by no known psychic or physical trauma and by no acute dietary excess of carbohydrate (the patient being on a constant diet in the sheltered environment of the metabolic ward). These observations imply either that the adrenal gland was unduly sensitive to normal day-to-day stimuli or that some defect in the hypothalamus or elsewhere was resulting in intermittent excessive release of glomerulotropin [153]. If the former alternative were correct it would be anticipated that the release of aldosterone would have been greater in these patients than in normal subjects following the same stimulus. Administration of glucose and insulin to normal young adult males has revealed increases of 17 hydroxycorticoids 17 ketosteroids and

aldosterone which were very similar to the changes seen in the patients with periodic paralysis [122]. Thus it appears that the increased carbohydrate utilization which results from the administration of glucose and insulin is a stimulus to adrenocortical hypersecretion in normal subjects. In addition, Conn et al [117] have found that the increase in urinary aldosterone resulting from sharp restriction of dietary sodium in normal people is seen also in patients with periodic paralysis. Thus the adrenal glands of patients with periodic paralysis probably possess no inherent abnormality in their responsiveness to physiologic stimuli. The implication is that intermittent hyperaldosteronism in periodic paralysis arises from an extraadrenal abnormality which stimulates hypothalamic release of glomerulotropin or from a primary defect in the hypothalamus characterized by intermittent "spikes" of glomerulotropin release.

It may be of some interest that glucose (150 gm) and insulin (20 units) in amounts which induced quadriplegia in a patient with severe periodic paralysis have been administered to a patient with severe primary aldosteronism due to an adrenocortical adenoma [7a]. No muscular weakness ensued. Aldosterone excretion did not increase but instead, fell from 100 to 30  $\mu$ g per day and the preexisting sodium retention was not aggravated in this patient in response to glucose and insulin. These findings indicate that the aldosteroma which proved to be present in one adrenal gland was not responsive to the physiologic stimuli to which normal adrenal glands respond. Certainly the responsiveness of the adrenal glands in patients with periodic paralysis to glucose and insulin administration and to other physiologic stimuli appears to be normal. On this basis as well as upon the basis of what scanty autopsy evidence is available one can eliminate with reasonable certainty, the likelihood that adrenocortical tumors are involved in the pathogenesis of periodic paralysis. The conclusion seems justified that in this disease there occurs periodically an excessive stimulus which results in the release of abnormally large amounts of aldosterone and that this stimulus arises by virtue of an abnormality which is not localized in the adrenal gland.

2 *Is there a primary defect in muscle membrane permeability?* Evidence can be marshaled for and against this proposal. Patients with periodic paralysis are regularly paralyzed by doses of 2 $\alpha$  methyl 9 $\alpha$  fluoro hydrocortisone (1 mg given once p.m.) or deoxycorticosterone acetate (12.5 mg b.i.d. intramuscularly). Such quantities of mineralocorticoids cause smaller decreases of serum potassium and no paralysis when administered to normal subjects. Figure 27.8 shows that a single dose of 2 $\alpha$  methyl 9 $\alpha$  fluorohydrocortisone (1 mg p.o.) produced a far greater fall in serum potassium in two patients than in two normal male subjects of comparable age and on the same diet. This suggests that an abnormality of membrane permeability to potassium is present in periodic

paralysis With respect to sodium however there is no evidence for the presence of a membrane defect in the kidney since the degree of sodium retention following this dose of 2 $\alpha$  methyl 9 $\alpha$  fluorohydrocortisone was not abnormal either at the time of onset of paralysis in the two patients

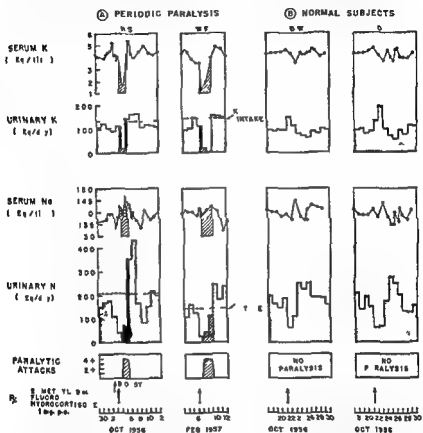


Fig 27.8 Effects of 2 $\alpha$  methyl 9 $\alpha$  fluorohydrocortisone on serum concentration and urinary excretion of sodium in normal subjects and in patients with periodic paralysis

or over the subsequent 3 days when compared with the changes in normal subjects. The paradoxical fall in urinary potassium excretion in the patients does not necessarily indicate the presence of a membrane defect since it might be explained by the more rapid and profound reduction of serum potassium as the result of more rapid sequestration of potassium presumably in the muscles in these individuals than in normal subjects (see Changes in Urinary Potassium above).

Immersion of one arm in cold water for 20 to 35 min may cause severe

weakness of the muscles of that arm [41, 59, 71, 90] without a concomitant fall in the serum potassium concentration in the weakened limb [41, 59]. This observation led McArdle [41] to conclude that the muscles themselves were abnormal in periodic paralysis and to postulate that the defect lay in the muscle membrane permeability. The abnormalities in muscle electrolyte composition (see under Chemical Composition of Skeletal Muscle, above) and other evidence confirm the conclusion that the muscle itself is abnormal but they do not eliminate the possibility that this abnormality might be secondary to a remote cause, such as a prior, chronic excess of aldosterone reaching the muscle through the circulating blood. It is quite possible, too, that cooling might inhibit the association of actomyosin in the muscles of the patient as it has been shown to do in vitro [126]. In the presence of abnormal electrolyte composition of the muscle this inhibitory effect of cooling might be sufficient to weaken or paralyze the muscle. Whatever the exact nature of the local effect of cold on muscle function is it might be significant that exposure to cold has precipitated paralysis in patients with primary aldosteronism [154] in whom composition of the muscles is deranged in the same way as it is in periodic paralysis (Table 27.3). The finding that in periodic paralysis the influx of potassium into muscle is not exaggerated but normal between attacks [20] may be cited as further evidence against any intrinsic defect of the muscle membrane.

On the other hand one could postulate a possible sequence of events based upon a primary abnormality with respect to potassium in bone muscle, or both in which (1) sequestration of potassium slowly increases to a critical value (2) this excites release of aldosterone, (3) the latter in the presence of adequate sodium produces a simultaneous increase in intracellular sodium and (4) abnormal muscle function or paralysis results from excessive intracellular concentration of cations.

#### OTHER HORMONAL CHANGES

##### *Follicle Stimulating Hormone (FSH)*

Cerny et al [114] have reported in a patient with familial periodic paralysis that urinary excretion of FSH was greatly increased to 80 and 400 rat units a day during two attacks and fell to below 20 rat units a day on two days between attacks. The low level between attacks was confirmed in two other patients.

##### *Thyroid*

Thyroid function has been normal in patients with sporadic periodic paralysis who had no clinical evidence of thyrotoxicosis [75]. In those patients who have had the clinical features of thyrotoxicosis associated with periodic paralysis there has been adequate laboratory evidence of hyperthyroidism in the form of elevated BMR, increased serum PBI

concentration and increased 1, 2, and 24 hr uptake of  $I^{131}$  by the thyroid gland [53, 55, 94, 116, 155]. The muscular disorder in these patients is similar to that found in the more usual euthyroid patients in that attacks are predominantly nocturnal, show a predilection for resting muscles, are always associated with a profound fall in serum potassium level, and can be prevented by a low sodium diet as well as by administration of the aldosterone antagonist SC8109 [155]. These observations and the findings that attacks of paralysis in patients with thyrotoxicosis are associated with an increase in aldosterone output [156] suggest that increased secretion of thyroid hormone may induce adrenocortical release of aldosterone. This possibility, the existence of which has yet to be demonstrated conclusively, would be consistent with the report of Shinosaki [10] that bouts of paralysis occurred with greater frequency in patients with periodic paralysis who were treated with large doses of desiccated thyroid. Since an increased output of ACTH, glomerulotropin, and FSH has been demonstrated or inferred before or during attacks, it might be speculated too that an excess of yet another tropic hormone, thyroid stimulating hormone, might be responsible for the thyrotoxicosis in these patients. There is at present no direct evidence to support this possibility.

## PATHOGENESIS

The available evidence is insufficient to establish conclusively the nature of the primary defect in periodic paralysis. However, the hypothesis to be advanced here is consistent with what evidence there is and might be useful in indicating where future studies would be expected to be fruitful.

Measurement of adrenal steroid excretion during spontaneous and induced attacks of paralysis has provided indirect and admittedly tenuous evidence that the primary defect in periodic paralysis might consist in the occurrence of intermittent excessive stimuli to the secretion of adrenocorticoids, particularly aldosterone. The possibility that these stimuli arise from the hypothalamus would be supported by the reported occurrence of periodic paralysis in patients with concomitant manifestations of hypothalamic disorders [51, 118, 157].

Intermittent mildly excessive secretion of aldosterone over a period of many years between attacks probably produces a slightly negative potassium balance by increasing urinary losses of potassium, as McArdle [47] has observed at least temporarily in a patient who ingested a diet containing low normal amounts of potassium. Thus, like all other losses of potassium from the body, would be expected to cause depletion of potassium in the skeletal muscle and its replacement by sodium. The shifts would produce the observed abnormalities in muscular electrolyte composition as well as in exchangeable body sodium and potassium.

between attacks for which no other explanation is at present available. In this way chronic low grade more or less intermittent excesses of aldosterone secretion probably set the stage in the skeletal muscle (and perhaps in the myocardium and even the smooth muscle as well) for the more severe sudden increases in aldosterone secretion which result from physical or psychic trauma ingestion of unusual amounts of carbohydrate foods, or perhaps 'spontaneous' hypothalamic discharge of glomerulotropin. Such sudden marked increases in the secretion of aldosterone as well as of hydrocortisone and other adrenocorticoids occur immediately before attacks of paralysis. The effects of these increased rates of steroid secretion may be recognized in the negative nitrogen balance increased uric acid excretion eosinopenia leukocytosis sodium retention and increased urinary excretion of pepsinogen and ammonia none of which probably plays any direct role in the pathogenesis of paralytic attacks. That paralytic attacks occur mainly at night and in resting muscles may result from the additive effects of rest and an excess of circulating aldosterone both promoting the rapid uptake of potassium by the muscles of which there is strong evidence during the attacks from a number of sources [19, 20, 21, 25, 96, 103]. That aldosterone does have a direct effect on skeletal muscle increasing its potassium content and reducing its sodium has recently been shown in acute experiments on mice [158] which await confirmation. The contrary opinion that aldosterone decreases muscle potassium and increases muscle sodium has been based largely upon indirect evidence from the effects of chronic aldosterone excess where renal wastage of potassium was probably responsible for the potassium deficiency and sodium excess in the muscles.

Under the influence of rest and acute hyperaldosteronism the influx of potassium into the muscle cells appears to be more rapid than the loss of sodium so that the muscles come to contain too large an amount and frequently too high a concentration of sodium plus potassium as has been shown in biopsy studies during paralysis (see under Chemical Composition of Skeletal Muscle above). It is probably the increased amount of sodium + potassium in the muscle cells which interferes with the association of actomyosin [126, 128] and in this way causes paralysis. Hyperpolarization of the muscle end plate and membrane resulting from the sudden rise in intracellular potassium concentration and the fall in serum potassium concentration might play a less important part in paralyzing the muscle. The fall in serum potassium concentration and in urinary excretion of potassium during the attacks probably result from rapid uptake of potassium by the muscle.

### DIFFERENTIAL DIAGNOSIS

When repeated attacks of flaccid muscular paralysis have occurred upon awakening in a young subject who appears to have been in perfect

health between attacks the only conditions which might be confused with periodic paralysis are *adynamia episodica hereditaria* and *hysteria*. *Adynamia episodica hereditaria* is readily differentiated by the clinical differences enumerated in Chap 28 and by the absence of a fall in serum potassium during an attack. *Hysteria* may be recognized by its characteristic psychologic features by the usual presence of sensory changes the persistence or even accentuation of reflexes the predilection for females and the retention of the muscular responses to electrical stimulation.

Less severe attacks causing weakness only may present more difficult diagnostic problems. The presence of muscular atrophy early in the course of the disease would favor a diagnosis of *progressive muscular atrophy* of spinal origin or one of the primary muscular dystrophies since in those few familial cases of periodic paralysis which manifest this sequela muscle wasting occurs only late in the course of the disorder [12]. The weakness of *myasthenia gravis* is characteristically least in the mornings and increases during the day it involves facial and ocular muscles early frequently lacks the intermittency of periodic paralysis responds dramatically to the administration of neostigmine and is not associated with hypokalemia though it may be improved by the administration of KCl. The myotonic components of *dystrophia myotonica* and *myotonia congenita* are absent in periodic paralysis. *Peripheral neuritis* produces sensory as well as motor impairment.

In a first attack of paralysis several other possibilities have to be excluded such as *cerebral lesions* (vascular accidents tumor etc) *spinal lesions* (tumor syphilis poliomyelitis) and *lesions of peripheral nerves* (polyneuritis Landry's paralysis Guillain Barré syndrome and diabetic neuropathy). The cerebrospinal fluid and concomitant neurologic abnormalities will usually enable these conditions to be differentiated from periodic paralysis without difficulty.

*Primary aldosteronism* even when associated with repeated attacks of paralysis is distinguished from periodic paralysis by the occurrence of hypertension and the persistence of hypokalemia between attacks.

## MORTALITY AND PATHOLOGIC FINDINGS

Talbott [49] has drawn attention to the fact that in about 10 per cent of the patients reported in the literature death has occurred during attacks of paralysis. A fatal outcome has been seen in sporadic as well as in hereditary cases and a particularly high rate of mortality has been recorded in some families such as Holtzapple's [60] in which six patients died in attacks of paralysis (Fig 27 2). The causes of death when related to the disease have been mainly *respiratory paralysis* [56 57 60 115] *respiratory infections* [46 60] *inhalation pneumonia* [10] *cardiac failure* [71] *shock* [51] and *excessive therapeutic venesections* [10 46 79].

*Autopsies* have been reported on a number of patients who died in or



out of attacks [7 10 16 46, 57, 60 79 90 114, 159] The changes of Graves' disease were seen in the thyroid of a patient who had hyperthyroidism [10] and evidence of cardiomegaly and venous congestion in patients who died in cardiac failure [114] Shinosaki [10] considered that the histologic appearance of the parathyroids was compatible with hyperfunction of these glands Unless this is a form of compensatory (secondary) hyperfunction it would be difficult to reconcile the invariable absence of hypercalcemia during attacks with primary hyperparathyroidism although the latter could explain the fall in serum phosphorus which usually occurs [13] The adrenal and other endocrine glands are not reported to have shown gross abnormalities Whether histologic studies were routinely performed on these glands in the studies reported is not stated

The only pathologic changes which appear to be specific for periodic paralysis are found in the skeletal muscles Goldflam [8] was the first to report the occurrence of histologic changes in the muscles in two brothers with periodic paralysis The lesion comprised an increase in the diameter of the individual muscle fibers rarefaction of the primitive fibrils and the formation of numerous vacuoles within individual muscle fibers The appearance of hypertrophied fibers in the muscles of patients with periodic paralysis has been confirmed by Schmidt [46] and Crafts [66] The more striking picture of vacuoles within the muscle fibers has attracted the attention of numerous authors who have debated whether these findings were a manifestation of real pathologic change or merely artifactual Goldflam [8] himself considered that the changes bore a causal relationship to the paralytic seizures and used their presence to exclude the possibilities that the paralysis resulted from neurosis or from a lesion of the central nervous system In agreement with Goldflam [8] Singer and Goodbody [14] were convinced that the vacuolar changes were not seen in sections of normal muscle and believed that the extraordinary fissuring seen on transverse sections of skeletal muscle was the result of some essential abnormality in the muscle fibres' Schmidt [46] found that some of the vacuoles were filled with granular masses which stained like glycogen with Best's carmine stain while others were filled with a homogeneous transparent shiny substance which did not take up the stains used He was emphatic that these changes were not seen in muscle from control subjects Neel [90] apparently unwilling to accept the verdict of his pathologist that the changes were artifacts pointed out that similar findings were absent from the muscles of his patient's unaffected sister as well as from the muscle of three patients of similar age who did not have periodic paralysis Zabriskie et al [71] confirmed the findings of Schmidt [46] that some of the vacuoles were filled with glycogen granules and shared the view that these lesions were indicative of pathologic change in the muscles Biernond and Daniels

[12] and Allott and McArdle [16] also observed the vacuoles but the former authors dismissed them as artifacts

Conn et al [23 24] showed by serial biopsies on a patient with sporadic periodic paralysis that the vacuoles were easily recognizable when the patient was paralyzed by glucose and insulin on a high sodium intake (Fig 27 9A) but disappeared completely (on biopsy from the same muscle) at a time when sodium depletion and a low sodium intake had reduced the sodium content of the muscle (see Table 27 2) as shown in Fig 27 9B. Specific stains failed to demonstrate the presence of either

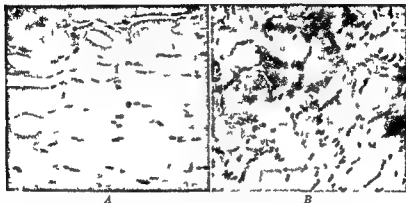


Fig 27 9 Photomicrographs of skeletal muscle from Patient R S showing vacuoles within fibers of muscle paralyzed on high-sodium diet (A) and disappearance of these vacuoles on low-sodium diet when paralysis could not be induced (B)

glycogen or lipid material in the vacuoles. These findings suggest that the vacuoles contained mainly sodium chloride and water and that the accumulation of excessive amounts of sodium and water within the muscle fibers might constitute an important step in the pathogenesis of periodic paralysis.

## GENETICS

From the numerous published pedigrees of patients with familial periodic paralysis it is evident that in most instances the defect is inherited as an autosomal dominant trait with complete penetrance [12 45 47 56 57 59 62 86 93 114 160 161]. From three of the larger kindreds collected from the literature and shown in Fig 27 3 it can readily be seen that one of the parents of each affected individual invariably suffered from the malady. A few authors have published pedigrees which indicate dominant autosomal inheritance with incomplete penetrance [40 44 46 60 89]. Three of these are illustrated in Fig 27 2.

It is not always clear whether the authors who described incomplete penetrance were able to verify the alleged absence of attacks in the parents of affected individuals by personal interviews with these parents.

At least in some instances evidence of incomplete penetrance is inconclusive, since it appears to be based upon the testimony of relatives whose contact with the allegedly unaffected individuals might well have been tenuous. A few patients have experienced their first attacks of paralysis in the fourth decade of life [10 12 93] or even at the age of 56 [9 61] and many authors [7 12, 47 56 90] have described members of affected families who in an entire life span experienced only a single attack or a few mild attacks all occurring within a brief period of time. It is likely, therefore as Dalinghaus [48] and Oliver et al [93] have suggested, that variability in the age of onset and in the severity and frequency of the paralytic seizures may explain why the disease appears to have skipped a generation in some families. If this suggestion is correct it might be expected that penetrance would appear to be less complete among females than among males since females are generally considered to suffer less severe and less frequent attacks than males [56 63]. That females do predominate in the apparently skipped generations [40 46 89] lends support to this hypothesis. The apparently normal parents of affected offspring might represent then *formes frustes* of the disease and might be expected to possess the fundamental underlying defect. Nobody has yet attempted to induce paralysis in such allegedly unaffected parents of sufferers from the disease by the repeated administration of glucose and insulin or of salt retaining adrenocorticoids.

One report has been published by Khan [78] describing the only Indian family in the literature in which the gene appears to have been sex linked and recessive. The eight affected individuals in three generations were all males and in each case the disorder was inherited from an unaffected female. No affected male transmitted the condition to his offspring. The females in the pedigree were summarily dismissed with the statement that all the sisters of the listed males were unaffected. Since the members of this family were Mohammedan peasants among whom the women usually refrain from social and even professional medical contact outside the family circle it is not unlikely that the author was unable to interrogate the females in person. The importance of a reliable history from the females of this family is evident when one reflects that if only one of the women had been found to have suffered from paralytic episodes the pedigree would have lost its seemingly unique features, becoming very similar to those published by Sagild [40] Brisset [97] and others, with a strong predilection for males because of incomplete penetrance in females. In the absence of more details this kindred cannot be considered to have established the existence of sex linked recessive inheritance in periodic paralysis.

Talbott [49] has implied that the existence of skipped generations in some families with periodic paralysis indicates recessive inheritance in those families. This implication is not generally considered valid as

Gates [162] has pointed out. Similarly Brisset's [97] claim to have shown recessive inheritance in periodic paralysis cannot be accepted. His claim rests entirely upon the finding of incomplete penetrance among the females in a pedigree where the occurrence of the malady in 8 of 17 adult descendants clearly indicates dominant inheritance.

There appears therefore to be no conclusive evidence in the literature which is incompatible with the view that familial periodic paralysis is inherited as an autosomal dominant trait with complete penetrance in most families and with apparently incomplete penetrance in others.

### *Sporadic Cases of Periodic Paralysis*

Patients with no family history of paralytic episodes have attacks which are identical with the hereditary type in their clinical manifestations and in their biochemical changes [21 49 163]. The age of onset is very similar (Fig. 27.1) the same stimuli provoke attacks and thyrotoxicosis may bring to light the underlying metabolic defect by precipitating attacks in both groups of patients [10 98]. While it is possible that inadequacy of information about the forebears may have been responsible for the failure to establish the truly hereditary nature of some allegedly sporadic cases, a strong case has been made for the existence of truly sporadic instances of periodic paralysis by the diligent studies of Gaupp and Kalden [163]. These authors traced and made personal contact with 186 out of the 261 living descendants of the grand parents of one of their patients and obtained adequate information from relatives and family physicians about another 50 of the descendants leaving only 17 per cent of the pedigree unaccounted for. Of the 236 individuals about whom adequate information was available none had any history at all suggestive of periodic paralysis.

Evidence for or against the existence of truly sporadic instances of periodic paralysis might be obtained by observing the responses of the siblings and parents of apparently sporadic patients to the administration of glucose and insulin or to adrenal salt retaining corticoids or simply to heavy meals followed by rest as Neel [90] suggested 30 years ago. No fall in serum potassium concentration and no paralysis resulted from the administration of 4 mg 9 $\alpha$  fluorohydrocortisone and 10 gm NaCl to the father and mother of a young man with sporadic periodic paralysis [70]. While this result will obviously need to be confirmed on the relatives of other sporadic patients, the consistency with which this stimulus will induce attacks of paralysis in affected patients supports the conclusion that the parents of this patient probably did not possess the genetic trait for periodic paralysis.

Of the 92 apparently sporadic cases of periodic paralysis which the authors have been able to trace in the literature [6 10 14 15 19 21 23-25 31 50-58 64-69 76 78 82-8, 87 88 91 92 94 98-103 105

111, 116 118, 163-170] only 7 have been females [10 19, 21, 51, 84 88, 99] The scarcity of female cases and the fact that their allegedly sporadic nature has sometimes been inconclusively documented [10, 19, 88, 99] or actually disproved by the transmission of the disorder to the offspring [21] allow the speculation that sporadic cases may not really occur at all in females It is possible that the apparently sporadic cases in males might result from inheritance of a recessive sex linked gene In the present state of ignorance however it cannot be stated with certainty whether sporadic instances of periodic paralysis result from recessive inheritance, whether they represent new mutations which will manifest dominant inheritance in succeeding generations, or whether their defect is acquired and not genetically determined in any way

## TREATMENT

The many forms of treatment that have been used in patients with periodic paralysis have included administration of Prostigmine, Meecholy, carbaminoyl choline [90] desiccated thyroid [32] ovarian substance [38] and use of an oxygen tent [116] and milk diets with the content of carbohydrate not higher than 10 per cent [137] Avoidance of high carbohydrate intake, particularly in the evening appears to be a reasonable recommendation to the patient in view of the ease with which glucose loads precipitate attacks

Potassium salts were used empirically for many years before the detection of hypokalemia during the attacks [14, 16 60] The administration of large doses (5 to 15 gm) of potassium salts (usually KCl) appears, in the opinion of most observers to shorten the duration of paralysis considerably when given during an attack [13 16 19, 21, 61, 89 95 114 116] Improvement in muscle function may occur within 15 min or may be deferred for 2 hr or more and has been attributed to a reversal of the abnormally rapid influx of potassium into the tissues [19] Of the few authors who have failed consistently to observe beneficial effects from the therapeutic administration of potassium some have probably used too small doses [15 98] and others may have been treating adynamia episodica hereditaria (which is aggravated by KCl) rather than periodic paralysis [31] Many observers have felt that the daily administration of KCl serves a useful prophylactic function [17 136] especially if KCl is administered at night [17] Others who have been enthusiastic about the effectiveness of KCl during attacks have expressed scepticism about its prophylactic efficacy and have even observed more frequent attacks when daily treatment with potassium chloride has been provided [16 21 25] Such a deleterious effect might conceivably result from stimulation of increased aldosterone output if these patients react like normal subjects to a high potassium intake [171 172]

The use of a 10 mEq sodium intake 'desalting' with diuretics (chlorothiazide, mercurials, acetazolamide), and quantitative replacement of potassium losses has been very effective in preventing both spontaneous paralysis and precipitation of attacks by the most potent of exciting stimuli including glucose and insulin and 2 $\alpha$  methyl 9 $\alpha$  fluorohydrocortisone. Conn et al [23, 24, 117] have made these observations in two severe and in two mild cases under strict supervision in a metabolic ward. One of the severely affected patients had thyrotoxicosis; after thyroidectomy he has required no sodium restriction or other treatment to avoid further seizures. The two patients who were mildly affected, experiencing attacks less than once a month, have both remained free of attacks at home over the past 6 months and 2 years respectively on a 10 mEq sodium diet (including salt free bread and cheese, no milk, and with supplementary calcium gluconate and vitamins). The patient with severe and frequent attacks of paralysis has frequently admitted his inability to adhere to the low sodium dietary regimen and has resorted to taking Randall's solution of potassium salts every day. He continues to experience many mild bouts of weakness and an attack of paralysis lasting several hours approximately once a week. It seems likely that there will be many such patients with severe forms of the disease who will fail to benefit from attempted sodium restriction because of dietary difficulties preventing strict adherence to the regimen. Under such circumstances the treatment will probably fail to reduce the sodium content of the skeletal muscle sufficiently to prevent paralysis resulting from a sudden influx of either sodium or potassium. Drugs (the steroidal spiro-lactones) are now becoming available. These inhibit the effects of circulating aldosterone and are successful in protecting patients from induced attacks of paralysis over the short periods of time that they have been used in these studies. It may be that chronic administration of these or similar agents may become a practicable form of therapy if the drugs prove to be well tolerated when administered over long periods of time.

Since in most patients there is a natural tendency for paralytic seizures to become less frequent and less severe after the age of 30 or 40 years, the mildly and moderately severely afflicted individuals may be treated satisfactorily with sodium restriction, intermittent use of oral diuretics plus potassium replacement, and perhaps aldosterone antagonists. On the other hand, periodic paralysis has proved to be lethal to 10 per cent of the sufferers [49], while many others have developed progressive muscular atrophy, often incapacitating. In families (such as that reported by Holtzapffe [60] and by Myers [61]) with a high incidence of severely afflicted patients, the untimed and as yet unrecommended use of subtotal adrenalectomy might be considered. This, however, remains in the realm of experimental medicine.

Patients who have coexisting periodic paralysis and thyrotoxicosis are

treated with complete success by elimination of the thyrotoxicosis, by appropriate use of propylthiouracil, subtotal thyroidectomy, or radioactive iodine [53, 55, 94, 98]

## SUMMARY

1 Periodic paralysis is a disease characterized by muscular paralysis without loss of consciousness speech or sensory modalities, in persons who enjoy good health and strength between attacks. Paralytic attacks commonly start between the ages of 7 and 21 years reach maximum intensity and frequency in the third decade of life and frequently tend to subside in middle or old age. The frequency of attacks varies from once in a lifetime to every night for many years. Males are more frequently and more severely involved than females.

■ Attacks of paralysis may be induced by a variety of "stresses" by factors which increase the rate of glucose utilization by rest and muscular relaxation particularly at night and by the administration of epinephrine desiccated thyroid corticotropin adrenocortical extract and mineralocorticoids.

3 Prodromata are usually absent. The flaccid paralysis with which the subjects characteristically awaken from sleep may be partial or complete but rarely involves facial and respiratory muscles. Tendon reflexes and muscular reactivity to electrical stimulation are abolished during paralysis. The frequent occurrence of cardiomegaly hypertension, bradycardia and arrhythmias and nausea vomiting constipation and reduced bowel sounds during paralysis of skeletal muscles suggests that the defect may involve the myocardium and smooth muscle as well. Severe thirst and increased fluid intake often precede attacks. Oliguria is usual during paralysis. Recovery is frequently heralded by severe diuresis and sweating.

4 Serum potassium concentration may be normal or lowered between attacks but always falls abruptly as an attack of paralysis develops. This fall is not due to external losses of potassium from the body. Urinary potassium output is reduced in parallel with and presumably because of the falling serum potassium concentration. An increased arteriovenous difference in serum potassium concentration before and during attacks has suggested that increased uptake of potassium by the tissues constitutes a cardinal feature of the paralysis. Determinations of the potassium and sodium content of biopsied skeletal muscle have confirmed a shift of potassium into the muscle without measurable change in intramuscular sodium concentration during paralysis.

5 Muscular strength is increased by potassium chloride administration but only after a delay when the arteriovenous difference in serum potassium concentration indicates that potassium is being lost from the tissues. Between attacks the potassium concentration in the muscles

is lowered and the sodium concentration is raised as in primary aldosteronism and other conditions of chronic potassium depletion. The exchangeable body potassium tends to be low and the exchangeable body sodium tends to be high. Retention of sodium and chloride usually precedes and accompanies both spontaneous and induced attacks. The high sodium content of the muscles probably predisposes them to paralysis. Since reduction of the sodium concentration, without change in the potassium concentration by 'desalting' with a low sodium diet, diuretics and potassium replacement frequently renders the muscles immune to the paralytic effects of glucose and insulin and of mineralocorticoid administration.

6 Attacks of paralysis are preceded by or associated with increased urinary excretion of aldosterone and of 17 ketosteroids and less consistently of 17 hydroxycorticoids. The increased glucocorticoid activity and 17 ketosteroid excretion are an integral part of the spontaneous paralytic attacks and do not occur in severe attacks induced by DCA and by ammonium glycyrrhizinate. Increased glucocorticoid secretion is not essential for the induction of paralysis.

7 Increase in aldosterone or aldosterone like activity is the only known factor which is common to practically all the means whereby paralysis may be induced; it therefore probably plays a significant role in the pathogenesis of paralysis. Sodium deprivation which abolishes most if not all of the physiologic effects of aldosterone affords protection from paralytic attacks.

8 The administration of glucose and insulin increases aldosterone output in normal subjects as in patients with periodic paralysis. It is probable that patients with periodic paralysis have neither adrenocortical adenomata nor any other intrinsic abnormality in their adrenal glands as the scanty autopsy evidence would confirm. Presumably periodic stimuli to adrenocortical hypersecretion of aldosterone—and other steroids—occur in patients with periodic paralysis.

9 The pathogenesis of paralytic seizures can be explained without postulating a primary defect in the muscle membrane but it is also possible that a defect in the normal partitioning of sodium or potassium between muscle and extracellular fluid might constitute the 'trigger' which stimulates periodic hypersecretion of aldosterone in this condition.

10 The defect in periodic paralysis might consist in intermittent excessive stimuli from the hypothalamus to the secretion of aldosterone and of the pituitary tropic hormones. This is suggested by the increased urinary excretion of follicle stimulating hormone reported during attacks of periodic paralysis, the similarity of the condition in thyrotoxic and euthyroid patients and the reported occurrence of periodic paralysis in patients with concomitant manifestations of hypothalamic disorders.

11 When death has occurred during a paralytic attack, it has usually



been due to respiratory causes. Autopsies and muscle biopsies have been largely unrevealing except for showing the presence of characteristic vacuoles within muscle fibers. Some of these are filled with granules which stain like glycogen; others appear to be filled with fluid.

12 In many instances periodic paralysis is clearly inherited as an autosomal dominant trait usually but not always with complete penetrance. A sporadic form almost certainly exists also. Families with periodic paralysis may have an increased incidence of migraine, epilepsy, and progressive muscular atrophy.

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## Chapter 28

### Adynamia Episodica Hereditaria

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*Jerome H. Conn and David H. P. Streeten*

The literature of the past 60 years contains a number of hints recognizable in retrospect of differences between the usual cases of classical periodic paralysis and the group recently described as adynamia episodica hereditaria [1]. Tyler et al. [2] were the first to appreciate that because the serum potassium level always fell during attacks in patients with the usual periodic paralysis, the consistent absence of hypokalemia in a family which they studied indicated the existence of at least two types of the disorder. These authors considered however that the clinical features in their patients were indistinguishable from those of periodic paralysis. This opinion was strongly supported by numerous published case reports of patients clinically identical with theirs described as having periodic paralysis. Sagild et al. [3] described a similar family and McArdle [4], referring to a family that he had studied, suggested the need for a separate name for this group of patients. Gamstorp and Mjones [1] were the first to draw attention to a number of clinical features such as the absence of changes in serum potassium level and in the electrocardiogram during attacks which prompted them to delineate from the main group of patients with periodic paralysis a group whose disorder they proposed to name adynamia episodica hereditaria.

In these patients the attacks of weakness usually started at an earlier age than in periodic paralysis, occurred more often by day than by night, were less widespread, less severe, and shorter. The attacks tended to be precipitated by hunger and by KCl administration and to be relieved by the ingestion of food. In a comprehensive review of the previous literature Gamstorp [5] found no less than 13 reports [2, 3, 6-16] of individuals or families whose clinical manifestations and normal potassium concentration during attacks strongly suggested the diagnosis of adynamia episodica hereditaria rather than that of classical periodic paralysis.



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manent muscular weakness occurred in 2 of the 138 patients described by Gamstorp [5].

### *Inciting Factors*

In all these patients attacks are most likely to occur during rest after severe muscular exercise [2 5-8 11-13 20]. Attacks may be brought on at will in some individuals by complete relaxation at any time of the day. Cold and dampness tend to bring on attacks [3 19] and it has been reported that epinephrine [18], emotional stress [2] 20 to 30 units of insulin [11] and glucose (200 gm) [18] will precipitate attacks. Tyler et al [2] have pointed out that a heavy meal at night will not cause any weakness within 2 hr but will frequently precipitate an attack the next morning. All these factors which precipitate attacks in adynamia episodica hereditaria may have a similar effect in periodic paralysis. In contrast with periodic paralysis however it has been reported that hunger frequently excites attacks of weakness in adynamia episodica hereditaria [5 19] while the ingestion of glucose or of a meal during the day appears to ward off an impending attack [5]. In contrast with periodic paralysis the administration of KCl in doses of 2 to 3 gm in children and 4 gm by mouth in adults almost always precipitates attacks [3]. One wonders whether or not KCl would precipitate attacks if such patients were maintained on diets very low in sodium.

## LABORATORY FINDINGS

Serum potassium has always been normal [2 10 12 18] or elevated [5 11] during attacks of weakness or paralysis whether the attacks occurred spontaneously or were induced by KCl administration.

The urinary potassium far from showing the sharp fall seen in attacks of periodic paralysis tended to rise during the seizures of adynamia episodica hereditaria [4 5 21 22].

The electrocardiogram showed lightly increased amplitude and a peaked appearance of T waves [3 5 20] reflecting the hyperkalemia during the attacks.

Exchangeable potassium is said to have been normal in three patients [22] though the actual figures have not yet been published.

The serum phosphorus has fallen in attacks [5] as in periodic paralysis. No abnormal findings have been encountered in serum albumin, globulin or cholesterol [15], sodium chloride,  $\text{CO}_2$ , Ca and Mg [3]. Hematocrit, fasting blood sugar [1], BMR [15], electroencephalogram and cerebrospinal fluid [3] have all been normal.

Blood eosinophil count has been observed to fall by more than 50 per cent in 19 out of 20 in attacks of spontaneous attacks and in 3 out of 11 attacks precipitated by KCl [3].

Four more reports have been found [17-19] of a condition which fulfills the criteria laid down by Gamstorp [5]. A recent report [20] of a family described as having *adynamia episodica hereditaria* has been published. It is not known whether the underlying pathogenic mechanism differs significantly in these two groups of patients but in view of the differences in clinical features, *adynamia episodica hereditaria* will be considered separately here.

## CLINICAL FEATURES

### *Age of Onset*

This is usually earlier in *adynamia episodica hereditaria* than in periodic paralysis, the condition is often recognized during early infancy. Thus among 108 patients in two families described by Gamstorp [5] the attacks started in the first 5 years of life in 50 per cent and before the age of 10 years in more than 90 per cent. This onset in the first decade of life contrasts with the onset in periodic paralysis which begins usually in the second or third decades as shown in Fig. 27.1

### *Description of Attacks*

Attacks occurred more frequently by day than by night [5, 6, 8, 10, 20] and 27 of the 138 patients described by Gamstorp [5] never experienced nocturnal seizures. Attacks occurred at night too [5, 6, 18] however, and tended to be more severe than the daytime seizures [20]. The duration of weakness or paralysis was very variable, lasting occasionally for 1 or 2 days but more frequently terminating within 10 to 45 min [5, 8, 9, 12]. One individual (G. D. H.) [19] described by Buzzard [6] in 1901 as having periodic paralysis has drawn attention to two main types of attacks in himself: (1) "ordinary attacks" of slight weakness involving a few or many muscle groups lasting up to an hour occurring as often as four times a day and resulting from such unpleasant experiences as hunger, cold, diarrhea or severe exercise and (2) "continuing attacks" with beaten, exhausted feelings in the legs lasting for several days. Many authors have described the ability of these patients to "walk off" attacks [2, 6, 8, 13, 21] as can patients with periodic paralysis. Severe sweating may occur during the seizures [6, 8, 20, 21]. A positive Chvostek sign was present in 25 per cent of Gamstorp's patients between attacks [5]. This sign was elicited in both the patients described by Kaplan et al. [20] but in one of these patients precipitation of an attack by potassium chloride administration was required to bring out the sign. Tyler et al. [2] have remarked upon the muscular build of a number of these patients. This often gives the appearance of the pseudo hypertrophy seen in childhood progressive muscular dystrophy. No deaths have been reported during paralytic seizures but per

manent muscular weakness occurred in 2 of the 138 patients described by Gamstorp [5]

### *Inciting Factors*

In all these patients attacks are most likely to occur during rest after severe muscular exercise [3 5-8 11-13 20] Attacks may be brought on at will in some individuals by complete relaxation at any time of the day Cold and dampness tend to bring on attacks [5 19] and it has been reported that epinephrine [18] emotional stress [2] 20 to 30 units of insulin [11] and glucose (200 gm) [18] will precipitate attacks Tyler et al [2] have pointed out that a heavy meal at night will not cause any weakness within 2 hr but will frequently precipitate an attack the next morning All these factors which precipitate attacks in adynamia episodica hereditaria may have a similar effect in periodic paralysis In contrast with periodic paralysis however it has been reported that hunger frequently excites attacks of weakness in adynamia episodica hereditaria [5 19] while the ingestion of glucose or of a meal during the day appears to ward off an impending attack [5] In contrast with periodic paralysis the administration of KCl in doses of 2 to 3 gm in children and 4 gm by mouth in adults almost always precipitates attacks [5] One wonders whether or not KCl would precipitate attacks if such patients were maintained on diets very low in sodium

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Blood eosinophil count has been observed to fall by more than 50 per cent in 19 out of 20 instances of spontaneous attacks and in 3 out of 11 attacks precipitated by KCl [5]

Urinary creatinine output has increased significantly on the day of the attack [3 17]

The electromyogram has revealed muscle action potentials which are always significantly shortened during paresis a change opposite to that seen in periodic paralysis (see Electromyographic and Chemical Changes in Muscle Chap 27)

All the patients studied have had an increased sensitivity to intra arterial injections of acetylcholine Thus 50 to 100  $\mu$ g acetylcholine produced a motor response in 5 patients, while 100 to 300  $\mu$ g was required in normal subjects This suggests a degree of hypopolarization of the muscle end plate both during and between attacks in adynamia episodica hereditaria

### PATHOLOGY

Tyler et al [2] have drawn attention to the presence of irregular clusters of nuclei along the more slender of the muscle fibrils and to the occurrence of vacuoles in the muscle Apparently these vacuoles are identical with those observed frequently in periodic paralysis (see Mortality and Pathologic Findings, Chap 27) Some of the vacuoles [2] contained unstained debris or appeared empty others contained granules which stained like glycogen with Best's stain However determination of the muscle content of glycogen has failed to show any abnormality

### PATHOGENESIS

There is little doubt that adynamia episodica hereditaria is a clinical entity which can be differentiated with reasonable ease from periodic paralysis Its strikingly earlier age of onset milder course and lack of incapacitating or fatal sequelae provide good practical grounds for differentiating it from periodic paralysis

On the other hand some of the other clinical differences may have been overemphasized While meals are said to improve symptoms during the day one wonders how conclusively this effect has been established in individuals whose weakness is mild and usually lasts for only 10 to 45 min without any treatment Furthermore it is heavy meals at night which are particularly likely to precipitate attacks in periodic paralysis and Tyler et al [2] have found precisely the same effect of heavy meals taken at bedtime in patients with adynamia episodica hereditaria viz attacks occurring early the next morning

The absence of a fall in serum potassium would be most unusual in severe attacks of quadriplegia occurring in patients with periodic paralysis but is certainly quite common in periodic paralysis during the mild spells of weakness which occur frequently between major bouts of paralysis This has been reported by Pudenz et al [23] Bickerstaff [24]

and others and is evident from Fig 27 II Moreover there is considerable doubt that KCl has any therapeutic effect in such spells of transient weakness in patients with periodic paralysis The beneficial effects in periodic paralysis of potassium even in severe attacks are seldom seen within 15 min of administration and may be delayed for 2 hr or more [25] Furthermore some patients with a clinical picture typical of adynamia episodica hereditaria are stated to have experienced relief from potassium administration despite the absence of a fall in serum potassium during the weakness [18]

Evidence suggesting a similar pathogenesis in the two conditions includes (1) the tendency in both conditions for attacks to be precipitated by a heavy meal at night [2] by glucose [18] or insulin administration [11] and by emotional stress [2] all of which can be expected to stimulate an increased adrenocortical output of aldosterone (see Nature of the Relationship between Transient Hyperaldosteronism and Paralysis Chap 27) (2) the fall in blood eosinophil count [5] which probably signifies increased adrenocortical secretion of hydrocortisone during seizures of adynamia episodica hereditaria as has been shown to occur in attacks of periodic paralysis (see Laboratory Findings Chap 27) and (3) presence of the identical pathologic lesion comprising vacuoles within the muscle fibers in both conditions [2 6]

Conclusive evidence on exactly how the pathogenic mechanisms of adynamia episodica hereditaria and periodic paralysis differ and in what respects they may be identical can be expected to result from future studies of muscle electrolyte composition adrenocortical activity and the ability to precipitate paralysis with potent salt retaining corticoids in patients with adynamia episodica hereditaria

## GENETICS

Dominant autosomal inheritance with complete or almost complete penetrance has been reported by most writers [2 3 7 8 12 13 17] and is shown in Fig 28 1 Penetrance was incomplete in one family [18] The incidence of the disorder among males and females in hereditary cases has been equal [21] though Tyler et al [2] found that the symptoms were more severe in males than in females A history of migraine or severe headaches has been encountered among relatives unaffected by adynamia episodica hereditaria [8 9] Apparently genuine instances of sporadic occurrence of this condition have been reported [9 10]

## TREATMENT

Administration of potassium salts either has proved of no avail [2 11 15] or has actually aggravated the symptoms [3 12] This finding is not sur

prising in view of the ease with which attacks have been precipitated by KCl. Premedication with glucose has prevented the precipitation of attacks by KCl [5].

Wolf [11] has reported very good results from the use of desiccated thyroid which given to seven patients completely protected them from attacks for  $1\frac{1}{2}$  years.

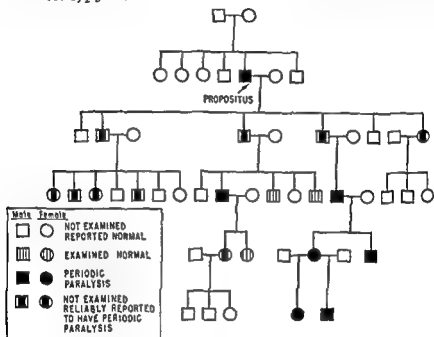


Fig 28-1 Genealogy of adynamia episodica hereditaria as exemplified by Gamstorp's family from Møller [5].

Intravenous calcium gluconate (5 to 20 ml 10 per cent solution) appears to have shortened attacks [1]. Cod liver oil (500 ml per week) is claimed to have prevented all attacks from 14 days after commencement of its administration [19].

Gamstorp et al [6] have recommended a regimen of moderate physical exertion, enough sleep at night and regular meals at short intervals during the day.

It is to be expected that newer information regarding the pathogenesis of adynamia episodica hereditaria and its probable relationship to periodic paralysis will be forthcoming and that rational therapeutic procedures will result.

## SUMMARY

1 Adynamia episodica hereditaria is delineated from classical periodic paralysis by the absence of the fall in serum potassium concentra-





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## Chapter 29

### Pseudohypoparathyroidism

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*Frederic C. Bartter*

Pseudohypoparathyroidism (PIIP) is a term introduced by Albright, Burnett, Smith, and Larson [1] to describe a syndrome in which the serum shows the abnormalities of hypoparathyroidism but which differs from 'idiopathic' or surgical hypoparathyroidism in that parathyroid extract has little or no effect in correcting the abnormalities. Whereas a typical case may present numerous distinctive features not found in idiopathic hypoparathyroidism, there is no general agreement as to the precise physical or biochemical criteria required to establish a diagnosis. Although this uncertainty has led some authors to include under pseudohypoparathyroidism cases with but a single feature and has even led a few to question the existence of the entity, an extensive and convincing body of literature now leaves little doubt that it exists as a unique syndrome and that it is a familial disease.

#### CLINICAL FEATURES

Patients with PIIP are almost invariably described as having short stature and round faces [2-3] (Fig. 29-1). They show, in addition, shortening of one or more metatarsal or metacarpal bones and shortening of the corresponding digits [4-6]. Clinical or radiologic examination may reveal areas of calcification or ossification in the skin or soft tissues. There may be exostoses typical of those seen in the syndrome of hereditary deforming chondrodysplasia [7-9]. A number of cases have been reported in siblings [2, 8-12]. If one includes in the significant family history the metatarsal and metacarpal changes resembling those of achondroplasia and the presence of exostoses, the evidence for positive family history is impressive.

The findings are not characteristic of idiopathic hypoparathyroidism. On the other hand, with the single exception of associated moniliasis [13], all the findings characteristic of idiopathic hypoparathyroidism have been reported in pseudohypoparathyroidism. These include both

tetanic and epileptic convulsions, fragility of the nails, thickness of the skull, hypoplasia of the enamel and of the roots of the teeth, cataracts and prolonged Q-T interval in the electrocardiogram. In both syndromes the serum alkaline phosphatase level is generally normal but may be elevated [2, 14, 15], the bones are generally thicker than average but may, indeed, be radiolucent [1, 3, 11, 16, 17] and there may be scattered areas of calcification in and about the region of the basal ganglia. Abnormally high serum phosphorus concentration, abnormally low serum calcium concentration and a very low urinary calcium excretion are characteristics of both.



Fig. 20-1 Face of patient G. H. with pseudohypoparathyroidism. (By permission of H. Etlrick et al. [6].)

Patients with PHIP are frequently mentally retarded [1-3] and the face is frequently expressionless, masklike, or suggestive of parkinsonism [3, 4]. In a number of reports the bones are described as showing increased trabeculation and coarse structure [11, 16, 17]. The first two features are difficult to evaluate quantitatively, and bone biopsy, which would allow better definition of the third, has been performed in only one atypical case [14].

As originally described [1] the syndrome differed in two cardinal features from idiopathic hypoparathyroidism: normal or indeed increased amounts of parathyroid tissue were demonstrated on exploration, and there was resistance to

administered parathyroid extract. The resistance can be demonstrated by two different types of test, the results of which may differ significantly.

In the first reported case [1, Patient I. S.] parathyroid tissue appeared to be normal in amount after 6 months of treatment with dihydrotachysterol. In two other cases [1, G. H. and S. M. B.] biopsy of the parathyroids showed hyperplasia. Figure 29-2 is a photomicrograph of the tissue removed from G. H. and S. M. B. The findings are in striking contrast to those reported for idiopathic hypoparathyroidism, in which parathyroid tissue has not been found despite careful search at autopsy on a number of occasions [10, 18]. MacGregor and Whithead note that in one reported case there was strong presumptive evidence for idiopathic hypoparathyroidism, but normal parathyroid tissue was found at autopsy [4]. In this patient a single normal serum calcium value was reported. The metatarsal and metacarpal bones were not described.

The literature contains a further report of a parathyroid biopsy in PHP [4]. No parathyroid tissue was found and the results were termed 'inconclusive' by the authors. Unfortunately biopsy has not been performed in any of the remaining cases thus far reported. Accordingly the diagnosis has been established on clinical grounds and with the use of



Fig. 212. Microscopic picture of parathyroid gland. A. A normal subject. B. patient G H [ ] with pseudohypoparathyroidism. C. patient S M B [•] with pseudohypoparathyroidism. Note the hyperplasia in (B) and (C). ( $\times 105$ )

parathyroid extract a procedure which as discussed below may give equivocal results.

The association of chemical hypoparathyroidism with hyperplasia of the parathyroids and resistance to parathyroid extract led Albright and associates to postulate an end-organ defect in PHP. They drew an analogy to Sebright Bantam roosters which develop the plumage of hens under androgenic stimulation.

### RESPONSE TO PARATHYROID EXTRACT

Response to parathyroid extract may be tested by observing the immediate changes in urine and serum phosphorus when the extract is

given intravenously, or by observing changes in serum calcium and phosphorus and in calcium and phosphorus balance when the extract is given intramuscularly over a period of days. The first type of test is generally done exactly as described by Ellsworth and Howard [20] thus it is possible to compare the quantitative results of one laboratory with those of another. The second type of test has been carried out under widely varying conditions of calcium and phosphorus intake and with differing doses of parathyroid extract so that quantitative evaluation of results from different laboratories is very difficult.

In the Ellsworth Howard test the patient is fasted and urine is collected for measurement of phosphorus content for 3 hr. Two hundred units of parathyroid extract are then given intravenously and urine and serum phosphorus are measured for a further period of 3 hr. In patients with idiopathic or surgical hypoparathyroidism urine phosphorus may rise to reach five to ten times control values and serum phosphorus may fall by 1.0 to 2.0 mg per 100 ml during the procedure. In normal subjects there may be an increase in urine phosphorus to two to four times control values and serum phosphorus may fall by 0.5 to 1 mg per 100 ml [1, 2]. In patients with PHP the urine phosphorus characteristically shows no change or a rise to not more than twice control values and serum phosphorus does not change.

The clinical application of this test presents four difficulties. In the first place parathyroid extract may lose its potency. To control this source of confusion the same batch of extract is often tested on a normal subject. In the second place normal subjects may show little change in urine or serum phosphorus even with potent extract [21]. This difficulty may be eliminated if it is possible to test the extract not on a normal subject but on a subject with known idiopathic hypoparathyroidism. In the third place some subjects with pseudohypoparathyroidism well documented in all other respects may show a rise of urine phosphorus to as much as five times the control values [2, 4]. This type of response may be a result of nonspecific action of the extract on the kidneys [22, 23]. It is known that commercially available extracts quite consistently increase renal plasma flow and some of them increase glomerular filtration rate and thus filtered phosphorus as well [23, 24]. This third source of confusion is eliminated if glomerular filtration rate measured during the Ellsworth Howard test shows no significant rise. Glomerular filtration rate has not generally been measured and hence many published data cannot be properly evaluated. A fourth source of confusion arises from reports of patients with steatorrhea and no other signs of PHP who show an elevated serum phosphorus level [25] instead of the lowered serum phosphorus level generally seen with steatorrhea. These patients may show no change of urine phosphorus during the Ellsworth Howard test [26]. This difficulty may be eliminated when patients with this syndrome

are tested with parathyroid extract over a period of days as discussed below

In the first report of the syndrome of PHP all three subjects were shown to be resistant to parathyroid extract given intramuscularly over 11 to 17-day periods as judged from negligible changes in serum phosphorus and calcium. Thus the confusion attendant upon nonspecific effects of the extract on renal function when administered intravenously was avoided and also the studies showed that in PHP there is resistance to the calcium raising property of parathyroid extract as well as to its ability to promote phosphate diuresis. These findings strongly imply that the effect of parathyroid hormone on bone [27] as well as on the renal tubules is diminished or absent in PHP. This conclusion derives also from the observation that most patients with PHP show normal to increased bone density; normal serum alkaline phosphatase values and no signs of osteitis fibrosa cystica such as loss of lamina dura, subperiosteal resorption or bone cysts. If parathyroid hormone is indeed secreted in excess in this syndrome, osteitis fibrosa would be expected unless the resistance involves the bones as well as the kidneys.

Resistance to parathyroid extract administered over a period of days has been used to aid in the diagnosis of PHP in only a few reported cases [1, 2, 4, 28]. In one case the initial response to parathyroid extract given intravenously was normal but the longer test confirmed the clinical impression of PHP by producing little change in serum phosphorus and only a transient change in serum calcium [2].

The effects of parathyroid extract given intravenously or intramuscularly may be influenced by previous administration. Acquired resistance to parathyroid extract has been well documented and may be associated with the presence of precipitating antibodies [29]. Accordingly resistance to extract in a previously treated patient may give a false-positive diagnosis of PHP.

The nature of the resistance to parathyroid hormone has not been clarified. Two groups of workers [2, 30] showed that the serum of patients with PHP would not interfere with the action of parathyroid extract given under standard conditions. In one study [2] dogs showed a normal rise in serum calcium with parathyroid extract given together with or previously incubated with serum from a patient with PHP.

## METASTATIC OSSIFICATION

Patients with PHP characteristically form bone in subcutaneous tissues and along fascial planes. This is true bone with normal histology. There is no evidence of osteomalacia (wide osteoid seam) and no evidence of osteitis fibrosa (osteoclasts and fibrosis). Ectopic bone formation is the cardinal sign of myostatic ossification syndrome in which

shortness of metacarpal and metatarsal bones closely resembling that found in achondroplasia is also a regular feature. This has suggested [2] that a common genetic defect may relate PHP and myositis ossificans progressiva.

### ABNORMALITIES OF BONE

Abnormalities of metacarpal and metatarsal bones form a cardinal feature of PHP. These bones are characteristically short and thick. They may show abnormal curvature as well. The first, fourth and fifth metacarpals are more frequently affected than the second and third [2]. There may or may not be early union of the epiphyses; the defect appears to be essentially a failure or limitation in linear growth at metaphyseal ends of bones exactly comparable to that seen in achondroplasia.

Exostoses have been noted in a number of cases [6-7]. These are indistinguishable from those seen in the syndrome of hereditary deforming chondrodysplasia [8] and as in that syndrome are frequently reported in the parents of patients with PHP. This finding lends support to the view that PHP represents a genetically determined defect.

### GENETIC ASPECTS

As noted above, PHP has a strong tendency to occur in more than one member of a family. This is a valuable aid to differential diagnosis since familial incidence is almost unknown in idiopathic hypoparathyroidism, even though there are many more reported cases of that disorder than of PHP. In the three siblings with idiopathic hypoparathyroidism reported by Sutphin and associates [13] there was associated moniliasis in all cases and all subjects also suffered from 'familial Mediterranean target oval cell syndrome'. In the family reported by Goldman and associates [52] hypocalcemia was found in three brothers who showed none of the other stigmata of PHP. The one patient who was studied showed a transient response to parathyroid extract given intravenously when a single hourly urine phosphorus reached four times the control value. In the family reported by Lachman [10] (Patients 57 to 59) the three siblings who were thought to have idiopathic hypoparathyroidism were short, mentally sluggish and childish and all had one or more short metacarpal bones. They were not tested for responsiveness to parathyroid extract but it seems probable that they suffered from PHP. If PHP is a genetically determined disorder and if it involves more than a single gene [2] one would expect to find incomplete cases. Although it is possible that the patients referred to above with steatorrhea, elevation of serum phosphorus level and resistance to parathyroid extract [20, 26] represent the abnormality in parathyroid function alone, this hypothesis cannot be tested directly until the nature of the resistance in PHP is more fully understood.

## PSEUDO PSEUDOHYPOPARATHYROIDISM

One type of incomplete PHP has been well documented. It is of interest that a number of reports have appeared describing cases with all the characteristic abnormalities of PHP except those associated with hypoparathyroidism [33-37]. They have been given the name of pseudopseudohypoparathyroidism. The abnormalities in these patients include the short metacarpal and metatarsal bones with brachydactyly, metastatic ossification, shortness of stature with roundness of the face, mental deficiency and indeed lenticular opacities despite absence of hypocalcemia or of any history thereof. These patients have normal serum calcium and phosphorus values and normal response to parathyroid extract. They do not show the calcification of the basal ganglia characteristic of idiopathic hypoparathyroidism and of PHP. This suggests that this manifestation is a result of the chemical abnormalities and is not under separate genetic determination. As in PHP, familial incidence has been reported in pseudopseudohypoparathyroidism. The presence of this incomplete syndrome lends strong support to the hypothesis that the fundamental disorder in PHP represents a genetically determined defect.

Recently Van der Werff ten Bosch [38] has reported that four of six female patients with this syndrome had gonadal dysgenesis. The nuclear pattern of three of them was chromatin negative for genetic sex.

## SUMMARY

1 Pseudohypoparathyroidism is a familial heritable disease in which the clinical and biochemical abnormalities of hypoparathyroidism are associated with skeletal abnormalities and metastatic ossification.

2 Patients are characteristically short with round faces, may have mental deficiency and show changes characteristic of achondroplasia in one or more metatarsal or metacarpal bones. True ectopic bone is found in skin and in fascial planes and there may be calcification of the basal ganglia.

3 The chemical abnormalities include hypocalcemia and hyperphosphatemia. Parathyroid extract produces little or no immediate phosphate diuresis and is ineffective in restoring serum calcium and phosphorus towards normal values. Parathyroid tissue is normal or hyperplastic. The disorder represents not a deficiency of parathyroid hormone but resistance to its effects.

4 Although only a single case has shown all the essential features (characteristic build, metacarpal defect, metastatic ossification, hypoparathyroidism, resistance to parathyroid extract in short term and long term courses and hyperplasia of parathyroid tissue) the syndrome seems to be a distinct clinical entity.



■ The syndrome of pseudo-pseudohypoparathyroidism appears to represent the same entity except that hypoparathyroidism and resistance to parathyroid hormone are absent

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5 The syndrome of pseudo pseudohypoparathyroidism appears to represent the same entity, except that hypoparathyroidism and resistance to parathyroid hormone are absent

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## Part Eight

### Diseases of Porphyrin Metabolism

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## Chapter 30

### The Porphyrins \*

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*Rudi Schmid*

Since the end of the nineteenth century porphyria has aroused the curiosity and interest of clinicians and investigators alike and has received wide spread attention in spite of its relatively infrequent occurrence. A continuing interest in disorders of porphyrin metabolism is largely due to two factors: (1) the unusual variety of clinical manifestations associated with these disturbances and (2) the relative ease with which very small amounts of porphyrins can be identified and estimated owing to their unique spectroscopic and fluorescent properties.

Porphyria has been the subject of innumerable clinical reports and of many biochemical investigations but only relatively recently has information accumulated regarding the nature and location of the metabolic defects which may account for the appearance of the disease. In the last 12 years with the advent of tracer techniques impressive progress has been made and much of the biosynthetic pathway by which porphyrins and heme are formed has been clarified. This better understanding of the normal pathways of metabolism provides an indispensable basis for the elucidation of the biochemical defects involved in the various types of porphyria.

The first authentic case of porphyria described in the literature was reported by Baumstark in 1874 [1] under the diagnosis of pemphigus leprosus. A craftsman with cutaneous photosensitivity and obvious liver disease was found to excrete a urinary pigment which was designated as uroburohaematin and which had unmistakably the spectroscopic and solubility properties of the compound now known as uroporphyrin. A few years later MacMunn [2] reported on five patients suffering from 'subacute rheumatism' or idiopathic pericarditis whose urine contained a red pigment which he named urohaematin.

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of clinical subforms with different manifestations such as abdominal colic peripheral neuropathy and pruritus psychosis and coma. In addition patients who excreted abnormal amounts and types of porphyrins including porphobilinogen but in whom clinical manifestations of porphyria were lacking were classified as having *latent porphyria* [14 16 18 19]. Such persons many of whom were blood relatives of patients with manifest porphyria were believed to exhibit the inherited metabolic abnormality but for unknown reasons they remained free of symptoms. It was recognized however that in such asymptomatic patients acute clinical manifestations could at times be precipitated by ingestion of relatively small amounts of sedatives such as barbiturates and sulfonal [14 20-22]. In these instances the drug undoubtedly converted a pre-existing latent porphyria into an acute form [14].

On the other hand acute porphyria was reported with increasing frequency in individuals who were addicted to sedatives but in whom no evidence of preexisting porphyria or familial occurrence of the disease could be elucidated [18 21 23 26].

Soon after 1888 the year of the introduction of sulfonal and related compounds into clinical medicine [20] massive haematoporphyrinuria was observed in two patients with chronic sulfonal poisoning [26]. By 1900 Taylor [27] was able to collect from the literature 34 cases of fatal sulfonal poisoning most of these patients showed a marked increase in haematoporphyrin excretion in the urine. Particularly striking was a patient with fatal porphyria described by Duesberg [24] who over a period of many years had ingested very large amounts of Sedormid (allyl isopropyl acetyl carbamide) and other sedatives. His urine exhibited high concentrations of uroporphyrin and at autopsy intense porphyrin fluorescence was demonstrable in the liver gallbladder and bile ducts [28].

The concept that in previously normal individuals sedatives of the sulfonal group could produce a porphyria like syndrome gained strong support from animal experiments. In rabbits it was observed that sulfonal poisoning regularly produced marked haematoporphyrinuria [6 20 29-31]. With more refined analytic methods Fischer and Duesberg [32] demonstrated uroporphyrin in the urine of such rabbits but a few years later Waldenstrom and Wendt [33] failed to confirm these observations. The question remained undecided until 1952 when it was discovered that in rabbits the sedative Sedormid also produced a syndrome which exhibited many biochemical similarities to human hepatic porphyria [34]. On a weight basis these animals were found to excrete more porphobilinogen and porphyrins than porphyric patients in relapse. Nevertheless certain biochemical differences between the hereditary human disorders and experimental Sedormid porphyria in animals were recognized. In the latter condition there was a precipitous fall in liver catalase activity



Although "urohaematin" undoubtedly was a porphyrin it is questionable whether MacMunn was actually describing uroporphyrin. Its solubility in chloroform and its spectroscopic properties which differed slightly from Baumstark's "urorubrohaematin" [3] suggest that MacMunn may have been dealing with the pigment which is today designated as coproporphyrin. In spite of the recognized slight spectroscopic differences Salkowski [3], Hammersten [4], Garrod [5], Stokes [6], Sallet [7], Anderson [8], Nebelthau [9] and others regarded these pigments for practical purposes as identical with hematoporphyrin<sup>1</sup> which Hoppe-Seyler [10] and Nencki and Sieber [11] had prepared from hemoglobin by the action of concentrated sulfuric acid. Garrod [5] discovered "haematoporphyrin or allied pigments" in the urine of 76 out of a group of 196 patients of a mixed hospital population. His conclusion that "haematoporphyrin" was an "almost constant constituent of urine in health and disease" reached far ahead into modern concepts of porphyrin metabolism. During the next two decades massive "haematoporphyrinuria" was observed in a large number of patients exhibiting such diversified clinical manifestations as photodermatitis, colicky abdominal pain, bizarre involvement of the nervous system or psychic disturbances.

### PROPOSED CLASSIFICATIONS

Guenther [12] was the first in attempting to arrange the reported cases according to certain recurrent symptom patterns. His classification distinguished between *haematoporphyrin congenita* and *haematoporphyrin acuta*, the latter including idiopathic and toxic forms. In addition he designated a few cases as *haematoporphyrin chronica*, a group which he originally considered as a separate entity but later tended to regard as mild and late forms of *porphyrin congenita* [13]. In such patients the photosensitivity and the excretion of red urine are indeed characteristic of what is now termed congenital (erythropoietic) porphyrin, yet these two conditions differ strikingly in their clinical course and particularly in the time of onset of the first symptoms. In 1937 Waldenström [14] proposed to substitute the term *porphyrin cutanea tarda* for Guenther's original "chronic porphyrin". In recent years the fundamental difference between *congenital photosensitive porphyrin* as a defect in the erythropoietic system and *porphyrin cutanea tarda* as a disturbance in liver metabolism has become well recognized [15-17].

Under the term *acute porphyrin* Waldenström [14] included a variety

<sup>1</sup> Hematoporphyrin is not found in nature but can be prepared from hemoglobin *in vitro*. In the earlier literature hematoporphyrin was used as a generic term to designate all naturally occurring porphyrins as well as those which can be derived from hemoglobin *in vitro*. Hematoporphyrin, hematoporphyrinuria and porphyrin were terms employed interchangeably.

From the above discussion it is evident that it may at times be difficult if not impossible to decide whether a patient is suffering from an inherited disorder or from an acquired disturbance of porphyrin metabolism. This is more frequently the case in porphyric patients exhibiting photosensitivity in whom liver disease is a prominent feature but similar difficulties may be encountered in patients with acute symptoms who have used excessive amounts of sedatives. The problem is further complicated by the fact that increased excretion of porphobilinogen and of uroporphyrins has been observed in patients with hepatic neoplastic and neurologic diseases in whom symptomatic manifestations of porphyria were absent [44-45].

The following discussion will be limited to those forms of porphyria for which a hereditary basis has clearly been established. For a more general consideration of disturbances in porphyrin metabolism the reader is referred to recent reviews by Watson [39], Rimington [46], Waldenström [10], Vannotti [47] and Bénéard [48].

## RELATIONSHIP OF PORPHYRINS TO HEMOGLOBIN METABOLISM

The name *porphyrin* appeared for the first time in the literature in 1871 when Hoppe Seyler [10] prepared haematoporphyrin from hemoglobin. A few years later Nencki and Sieber [11] identified haematoporphyrin as a derivative of hemin, the prosthetic group of hemoglobin. Although a number of investigators [2, 3, 6, 7, 31] recognized the slight spectroscopic differences between hematoporphyrin and the porphyrins excreted in the urine for practical purposes these pigments were considered as identical. This led to the widely accepted but erroneous belief that the urinary porphyrins are derived from breakdown of hemoglobin [2, 6, 19, 47, 49-51] although several observers including East and Weiss [31], Garrod [5] and later Liebig [52] raised doubts as to the validity of this concept. It was not until 1920 that direct proof became available for the independence of porphyrin excretion from hemoglobin breakdown. Fischer and his coworkers [53] found significant structural differences between hematoporphyrin and the porphyrins present in the urine. More important still was the finding that the urinary porphyrins belong in part to an isomeric series different from that of hemoglobin protoporphyrin (Fig. 30-1).<sup>a</sup> These and other observations led Fischer to sug-

The classification of porphyrin isomers is based on the four synthetically prepared isomers of etioporphyrin designated as I, II, III, and IV. In nature only porphyrins belonging to isomer series I and III are found. The etioporphyrin of hemoglobin and of other heme proteins has the basic structure of a porphyrin of type III isomer. However, since the  $\beta$  positions of the pyrrole rings are substituted by three different radicals, the total number of possible isomers for protoporphyrin is 15. The protoporphyrin occurring in all known heme proteins is designated as isomer 9a and corresponds to the basic structure of etioporphyrin in III.

[32] the urinary porphyrins were almost exclusively of the type III isomer [34] and the concentrations of proto- and coproporphyrin in the liver and bile were greatly increased [36]. On the other hand, no increase in the urinary excretion of  $\delta$ -aminolevulinic acid was observed [37] although porphobilinogen was present in high concentrations [36].

These animal experiments support the concept that in persons who habitually ingest large amounts of sedatives, a toxic form of porphyria may develop. This is distinct from latent hereditary porphyria in which relatively small amounts of sedatives may precipitate an acute attack. Since detailed studies of human toxic porphyria have not been reported it is not known how this condition differs from the hereditary forms of porphyria. The chemical findings in experimental Sedormid porphyria suggest that the metabolic derangement in toxic porphyria may be quite different from that in the genetically controlled disorders.

The concept that some forms of human porphyria may not have a hereditary basis has recently gained additional support from studies of patients with cutaneous porphyria. The term "porphyria cutanea tarda symptomatica" has been proposed by Waldenstrom [16] to describe patients in whom outspoken liver disease is associated with photosensitivity and marked elevation of porphyrin excretion. In this group he included some of the patients who previously had been reported as having porphyria cutanea tarda by Brunsting [33], Watson [39], Bolgert et al [40], Schmid et al [41] and Barnes [42] and in whom liver disease was a prominent feature. Waldenstrom believes that in such patients the liver disease usually of alcoholic or nutritional origin is the primary disorder and that the disturbance in porphyrin metabolism is merely a secondary manifestation [16]. An interesting case of seemingly acquired cutaneous porphyria was recently reported by Tio Tiong Hoo [43]. In an 80-year-old woman photosensitivity and marked porphyrinuria had gradually developed over the course of a year, when she was discovered to have a large benign liver cell adenoma. On resection this was found to contain large amounts of proto-, copro- and uroporphyrin; the surrounding liver tissue was normal. Following the operation the cutaneous manifestations disappeared and the porphyrin excretion returned to normal values. This seemed to indicate that the defect in porphyrin metabolism was limited to the hepatic tumor.

Although in most of the above-discussed patients there is no positive family history nor evidence suggesting a preexisting latent porphyria the possibility cannot be excluded with certainty that a predisposition to porphyria may have been inherited and the acquired liver disease may merely have served to bring out the clinical and chemical manifestations of this disturbance. Moreover it should be noted that several genetically distinct forms of porphyria exhibiting cutaneous manifestations have been described in which the hereditary nature of the disorder has clearly been established. These will be discussed later.

zymes such as cytochromes catalase and peroxidase [60]. Although the amounts of these heme proteins in the organism may be quite small as compared with hemoglobin, some of them may have a more rapid turnover than hemoglobin. For liver catalase, for example, the half life has been estimated to be only a few days [30, 61], a rate of turnover which requires the daily synthesis of a considerable amount of protoporphyrin. It is therefore conceivable that metabolic abnormalities resulting in excessive formation and liberation of porphyrins and of porphyrin precursors may occur in any tissue of the body, but that the liver is of particular importance as the site of such disturbances. In fact, with the exception of congenital erythropoietic porphyria, the liver seems to be the major site of the metabolic derangement in most forms of human porphyria and in experimental porphyria produced by Sedormid. This has led to the proposal to classify these disturbances under the group name of *hepatic porphyria* [18, 39, 41].

## BIOSYNTHESIS OF PORPHYRINS AND OF HEME

Little was known prior to 1946 about the chemical building blocks from which the body synthesizes porphyrins and heme. In that year Shemin and Rittenberg [62, 63] observed that after feeding isotopically labeled glycine to man or animals, the concentration of the label in the circulating heme of hemoglobin rose rapidly, remained more or less

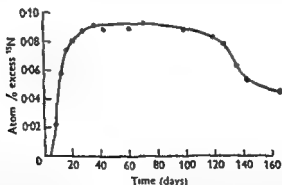


Fig. 30-2. Concentration of  $N^{15}$  in hemoglobin heme of a normal subject given 10 gm glycine- $N^{15}$  (31.65 atom per cent excess  $N^{15}$ ) during the first 4 days of the experiment (By permission of C. H. Gray [30]).

steady for about 100 days and then rapidly declined (Fig. 30-2). The disappearance of the label from the circulating heme was accompanied by a marked though temporary increase in the fecal excretion of labeled urobilinogen [64, 65] (Figs. 30-2, 30-14). These observations permitted the conclusions that (1) glycine is a specific precursor of heme protopor-

gest that in congenital (erythropoietic) porphyria porphyrins of the type I isomer are synthesized along with and parallel to hemoglobin formation in developing erythroid cells of the bone marrow. Isser considered this "atavism" as a partial regression into a phylogenetically and ontogenetically more primitive phase of development. As will be seen later, this concept is to a large extent in agreement with more recent studies.

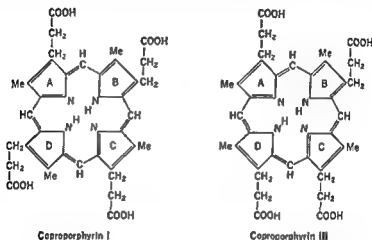


Fig. 30-1 : Coproporphyrin of type I and type III isomer series. In porphyrins of type III isomer, the sequence of the side chains in the  $\beta$ -positions of pyrrole ring D is reversed. Me = methyl.

Although as a result of these observations it was generally accepted that porphyrins may be formed along with hemoglobin in the bone marrow, it soon became apparent that this hypothesis was not applicable to patients with acute porphyria and porphyria cutanea tarda. On the basis of extensive clinical and necropsy studies, Waldenström [64] came to the conclusion that there must exist a fundamental difference in the pathogenesis of the various forms of porphyria. He reasoned that "the porphyrin must either be an anomalous product on the way to hemoglobin which is then excreted (or) the other possibility is to try to find another source from which porphyrin might appear." The nature of this other source was suggested by autopsy studies [28, 35, 41, 47, 65-69] and by analysis of biopsy material obtained from patients with porphyria [41]. These investigations were supplemented by animal experiments as discussed in the preceding section.

As a result of these observations it became increasingly clear that not only the erythropoietic system but also other organs and particularly the liver may be important sites of porphyrin formation. This is not surprising since most mammalian cells can synthesize the porphyrins which are required for the formation of essential heme-containing en-

[70-76] With methyl labeled acetate for example highest labeling of heme protoporphyrin is observed in the carbon atoms 6 and 9 (Fig. 30-4). The activity is present in positions 4, 8, and 3 [76]. Moreover there is equal labeling each to each in the position pairs 6 and 9, 4 and 5, and 3 [76]. The labeling pattern obtained in protoporphyrin synthesized from succinate 1-4  $C^{14}$  is pictured in Fig. 30-4 [79].

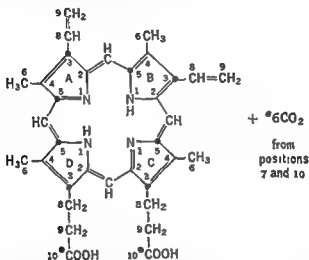


Fig. 30-4 The labeling pattern obtained in protoporphyrin synthesized from succinate 1-4  $C^{14}$ . Carbon 1, which gives rise to the CO is the carbon of the original carboxyl group attached to carbon 6 (By permission of D. Shemin [10]).

These findings indicate that the biosynthesis of the protoporphyrin molecule requires 8 moles of glycine and 8 moles of a four-carbon intermediate of the tricarboxylic acid cycle most likely succinate [70-74]. The possible ways by which succinate and glycine could combine to form a pyrrole unit are limited by the finding that the  $\alpha$ -carbon atom of glycine is always equally utilized for both the pyrrole ring carbon atoms and the methene bridge carbon atoms [70-77].

The above and other observations led Shemin and his coworkers [70-78] to suggest that the intermediate which is formed by condensation of glycine and succinate is the five carbon aminoketone  $\delta$ -aminolevulinic acid (ALA) (Fig. 30-3). With the use of ALA 5  $C^{14}$  in a system of hemolyzed duck red cells the position of this aminoketone as a true intermediate in porphyrin biosynthesis has been well documented [79-81]. Not only was the labeling pattern of the  $\delta$  carbon atom of ALA identical with that of the  $\alpha$ -carbon atom of glycine [70-79] (Fig. 30-3) but the heme synthesized from an equimolar amount of ALA 5  $C^{14}$  was about sixty five times more radioactive than heme synthesized from glycine

phyrin, (2) hemoglobin of the circulating erythrocytes remains outside the general metabolic pool of protein interchange (3) the majority of normal red cells have a mean life span of about 120 days (4) a large fraction of the fecal urobilinogen is derived from the breakdown of circulating red blood cells, and (5) the metabolites of heme protoporphyrin are not reutilized for the synthesis of hemoglobin in newly formed erythrocytes

Additional studies carried out in various laboratories showed that blood from ducks and chickens which contains nucleated erythrocytes [66

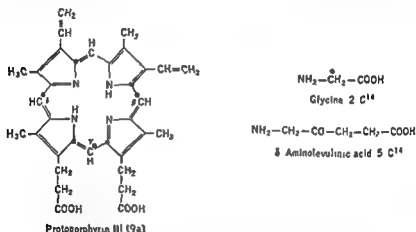


Fig 30-3 The carbon atoms of protoporphyrin derived from the  $\alpha$ -carbon of glycine and the  $\delta$ -carbon of  $\delta$  aminolevulinic acid (ALA) (By permission of D. Shemin [6])

67] and blood from rabbits which had been made anemic to produce a high percentage of reticulocytes [69] is able to synthesize labeled heme from glycine  $\text{C}^{14}$  in vitro. The availability of these systems greatly facilitated subsequent studies which revealed that all four nitrogen atoms [62-69, 70] and eight carbon atoms of the protoporphyrin molecule are derived from glycine [70-72] (Fig 30-3). Four of the carbon atoms are still attached to the nitrogen atoms of glycine while four are not forming the methene bridges which link the four pyrrole rings to form the porphyrin ring (Fig 30-3). All eight carbon atoms are derived from the  $\alpha$  carbon atom of glycine while the carboxyl carbon of glycine is not utilized for any of the carbon atoms of porphyrin [72, 73].

The remaining 26 carbon atoms of protoporphyrin arise from intermediates of the tricarboxylic acid cycle [70, 74]. Acetate labeled either in the methyl or in the carboxyl group = ketoglutarate-5  $\text{C}^{14}$  or  $\alpha$  keto glutarate-1  $\text{C}^{14}$  or citrate 1, 5  $\text{C}^{14}$  lead to characteristic and predictable labeling patterns which strongly suggest that the same four-carbon chain is utilized in the formation of both sides of all four pyrrole rings

reactions all involving the transformation of glycine [70]. The formation of porphyrins is only one of these various metabolic pathways

### BIOSYNTHESIS OF $\delta$ AMINOLEVULINIC ACID (ALA)

The elucidation of the exact mechanism by which ALA is formed by condensation of glycine with active succinate was a difficult task. Shemin's observations indicated that the system required some particulate matter present in nucleated red cells of birds or in reticulocytes of mammals [70-78-79] but absent from mature mammalian erythrocytes [88]. The findings have now been confirmed and extended [83-86] but the nature

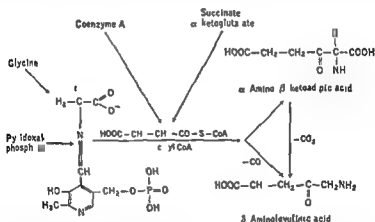


Fig 30-6 Condensation of succinyl CoA with glycine in the presence of pyridoxal phosphate (Adapted from Hikiuchi et al [87])

of the cellular particles has not been conclusively established [80] (Fig 30-6). In more recent studies Hikiuchi et al [87], Gibson [88] and Sawyer and Smith [89] have observed enzymatic synthesis of ALA from succinyl CoA in particle-free extracts obtained from *Rhodospseudomonas spheroides* or *Rhodospirillum rubrum*. A forty to eighty fold purification of the soluble enzyme has been achieved [87].

Shemin and Wittenberg's original suggestion that the 'active' four carbon intermediate may be succinyl CoA [74-76-77] has gained strong though indirect support from studies of heme and porphyrin formation in pantothenate deficient ducks [90] and in *Tetrahymena vorax* [91]. The more recent studies by Gibson, Laver and Neuberger [84], Shemin et al [87], Granick [85] and Brown [92] have provided conclusive evidence that succinyl CoA is indeed the intermediate which condenses with glycine. The succinyl CoA can be derived from  $\alpha$ -ketoglutarate by the action of  $\alpha$ -ketoglutarate dehydrogenase [83-84] or it may be formed from succinate in the presence of ATP [84-87-92] (Fig 30-6).





first by Westall [102]. The soluble enzyme ALA dehydrase has been purified from a number of sources including duck erythrocytes [98], chicken erythrocytes [103] or liver [99-100] and spinach leaves [104]. Reduced glutathione is required for activation of the enzyme system [99-100-105]. SH group inhibitors such as iodoacetamide and *p*-chloro mercuribenzoate have an inhibitory effect [100]. The latter can be overcome by the addition of reduced glutathione [99-100]. In addition marked inhibition of ALA dehydrase has been noted with the chelating agent ethylenediaminetetraacetic acid a finding which suggests that a metal may be essential in this enzymatic step. This has recently been

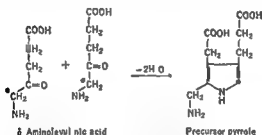


Fig 30-7 Condensation of 2 moles of  $\delta$  aminolevulinic acid to form 1 mole of precursor pyrrole (porphobilinogen). The carbon atoms bearing the labels are the original  $\alpha$ -carbon atoms of glycine (By permission of Dr Shemin [6]).

confirmed by the observation that ALA dehydrase contains copper [106]. Furthermore ducklings deficient in copper were found to exhibit reduced ALA dehydrase activity in liver and erythrocytes [106]. Similar observations have been reported in copper-deficient chicks [107] and copper deficiency has been shown to lead to hypochromic anemia.

Two moles of ALA are required for formation of 1 mole of PBG [70-98] (Fig 30-7). Heme synthesized from labeled ALA has twice the specific activity of that which is formed from an equimolar amount of PBG with the same radioactivity [70-98]. These findings confirmed the earlier reports by Falk, Drechsel and Rimington [108] and by Bogorad and Granick [109] that PBC is a specific precursor and an obligatory intermediate in the biosynthesis of porphyrin and of heme.

#### BIOSYNTHESIS OF UROPORPHYRINOGEN

In considering the conversion of PBC to porphyrins and to heme two important findings have to be taken into account. In earlier studies it had been assumed that simple linear condensation of four pyrrole units would lead to formation of the next metabolic intermediate uroporphyrin; the latter would then undergo stepwise decarboxylation to yield protoporphyrin. It soon was discovered however that this simple scheme was not correct. In a variety of systems which were able to convert PBC to

The condensation of glycine with succinyl CoA requires the presence of pyridoxal phosphate. The requirement for pyridoxal was demonstrated along three different lines of evidence. In blood of pyridoxine-deficient ducklings Schulman and Richert [90] observed reduced heme synthesis from glycine C<sup>14</sup>, a defect that could be corrected by the addition of pyridoxal phosphate. This finding is in line with earlier observations by Wintrobe [93] that pigs deficient in vitamin B<sub>6</sub> produced small pale red cells with a low content of hemoglobin and of free protoporphyrin. In vitro compounds known to inhibit enzymes which contain pyridoxal phosphate [94] have been shown to reduce the formation of ALA [83, 84, 87] and of porphyrins [89, 95]. Marked inhibition was obtained with L-penicillamine, nicotinic acid hydrazide, cyanide, L-cysteine and p-chloromercuribenzoate. Finally, biosynthesis of ALA [83, 84, 87, 97] and of porphyrin [89, 90, 91] was found to be enhanced by the addition of pyridoxal phosphate in vitro. These findings indicate that pyridoxal phosphate is essential for the condensation of glycine with succinyl CoA and it appears likely that the cofactor is firmly bound to the condensing enzyme (Fig. 30-6) [83, 84, 87].

The primary condensation product is probably  $\alpha$ -amino  $\beta$ -ketoadipic acid [70, 78, 80] (Fig. 30-5, 30-6) but the ease with which this compound undergoes spontaneous decarboxylation has prevented its isolation [84]. However, the finding that in the intact rat injection of the diethyl ester of this aminoketone resulted in urinary excretion of porphobilinogen provides at least indirect evidence to support this hypothesis [160].

The biosynthetic pathway of ALA formation is summarized in Fig. 30-6. Aerobic reactions which are inhibited by dinitrophenol [85, 96] convert  $\alpha$ -ketoglutarate [83, 84] or succinate [87, 92] to succinyl CoA. An enzyme which requires pyridoxal phosphate as a cofactor catalyzes the condensation of succinyl CoA with glycine to form  $\alpha$ -amino  $\beta$ -ketoadipic acid. By decarboxylation the latter is converted to ALA. The initial reaction of this pathway requires a functioning tricarboxylic acid cycle and thus is oxygen dependent; the condensing enzyme system is operative under anaerobic conditions. In mature mammalian erythrocytes the absence of a functioning tricarboxylic acid cycle obviously precludes formation of succinyl CoA and hence of ALA.

#### BIOSYNTHESIS OF PORPHOBILINOGEN (PBG)

With the establishment of ALA as an obligatory intermediate in porphyrin and heme biosynthesis, studies of the subsequent enzymatic steps were greatly facilitated. ALA dehydrase was found to catalyze condensation of 2 moles of ALA to form a precursor pyrrole [97-100] (Fig. 30-7). The theoretical formulation of the structure of this pyrrole [78] yielded the same structure as porphobilinogen (PBG) [101], a chromogen excreted in the urine of patients with acute porphyria [14] and isolated

type III isomer is as yet incomplete sufficient information is available to formulate a reasonable working hypothesis [113] (Fig 30-9) Incubation of PBG with an enzyme system obtained from *Chlorella* [109] or from red cell hemolysates [124-125] results in formation of porphyrins of type III isomer However if such a system is heated for 15 min at 55 C it is changed in such a way that only porphyrins of type I isomer are formed This finding suggests that the enzyme porphobilinogenase may have more than one component Indeed Bogorad [116-117] and Hoare and Heath [114] succeeded in preparing two separate enzyme fractions PBG deaminase and uroporphyrinogen isomerase It appears that one of the actions of PBG deaminase is to remove the amino group and to condense individual monopyrroles to form polypyrrylmethanes (Fig 30-9) [105-117] The enzyme is relatively heat stable and if acting alone converts PBG to uroporphyrinogen I [116] Inhibition has been observed with silver and mercury ions and with formaldehyde [116] The latter may be of biologic importance as a possible regulatory mechanism promoting formation of uroporphyrinogen III since formaldehyde is formed in the deamination of the aminomethyl group of PBG [126]

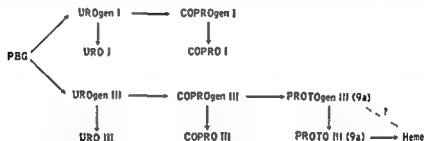
If uroporphyrinogen isomerase from wheat germ is added to a system containing PBG and PBG deaminase the reaction product is uroporphyrinogen III [117] (Fig 30-9) Since by itself uroporphyrinogen isomerase reacts with neither PBG nor uroporphyrinogen I [113-117] it is clear that its enzymatic function is dependent on some effect of PBG deaminase on PBG It seems reasonable to assume that the isomerase acts by combining PBG with a noncyclized intermediate which is produced by the action of PBG deaminase on PBG [117] Such an intermediate may be a di- or tripyrrylmethane [105-117] (Fig 30-9)

#### METABOLISM OF UROPORPHYRINOGEN

The further metabolism of uroporphyrinogen can proceed along two separate lines it may undergo either a stepwise oxidation to uroporphyrin or a stepwise enzymatic decarboxylation to porphyrinogens which exhibit less than eight carboxyl groups [112-114 118-124] (Fig 30-10) The oxidative process leads to uroporphyrin which is a side product and does not serve as a substrate for the enzyme uroporphyrinogen decarboxylase Nonenzymatic oxidation of uroporphyrinogen can occur by photocatalytic autoxidation a process activated by the product uroporphyrin (Fig 30-10) [105] Photooxidation is partially inhibited by glutathione ■ mercaptoethylamine cysteine or sodium ulfite and is markedly reduced under strictly anaerobic conditions or by exclusion of light [105-116] On the other hand ferric ion at acid pH catalyzes the photooxidative process [105]

The conversion of uroporphyrinogen to uroporphyrin proceeds stepwise with formation of cyclized tetrapyrroles which exhibit an oxidation

heme or to porphyrins with less than eight carboxyl groups, uroporphyrin was ineffective as a substrate and could not replace PBG [70 110 111]. The observations suggested that the true intermediate formed from PBG is not uroporphyrin but its reduced form uroporphyrinogen. Indeed Neve and his coworkers [112] found that in a hemolyzed red cell system incorporation of  $^{59}\text{Fe}$  into heme was markedly stimulated by the addition of uroporphyrinogen but not by uroporphyrin. Additional studies [104 113-118] have clearly indicated that the biosynthetic pathway from PBG to heme proceeds over a series of porphyrinogens which are colorless reduced porphyrins containing six additional hydrogen



**Fig 30-8 Metabolism of porphobilinogen.** Only porphyrinogens of type III isomer are intermediates in the biosynthesis of heme. Porphyrins of type III isomer and porphyrinogens and porphyrins of type I isomer are side products not utilized for heme synthesis. PBG porphobilinogen, UROgen uroporphyrinogen, COPROgen coproporphyrinogen, PROTOgen protoporphyrinogen, URO uroporphyrin, COPRO coproporphyrin, PROTO protoporphyrin.

atoms [119]. The oxidized porphyrins instead of being true intermediates are merely by products resulting from irreversible oxidation of the corresponding porphyrinogens (Fig 30-8) [120].

A second point of importance is that the protoporphyrin of hemoglobin and of other heme containing proteins belongs to the type III isomer series (protoporphyrin 9a) [53] (Fig 30-1). Heme proteins containing a porphyrin of type I isomer have never been found in nature [121]. Thus, any biosynthetic scheme proposed for the formation of heme must include a mechanism which permits synthesis of type III isomer porphyrins. Since in nature isomerization of porphyrins by ring opening and reclosure in a different position does not appear to occur [113] the isomer type formed must be determined at the time of the original ring closure (Fig 30-1). The exact mechanism by which this occurs is still in doubt. Several suggestions have been advanced [70 105 109 122] but they will not be discussed in detail. It may suffice to state that PBG can condense to uroporphyrin by nonenzymatic means and that under such conditions mixtures of uroporphyrin isomers are obtained [122 123].

Although knowledge regarding the precise mechanism and nature of some of the intermediates involved in the formation of porphyrins of

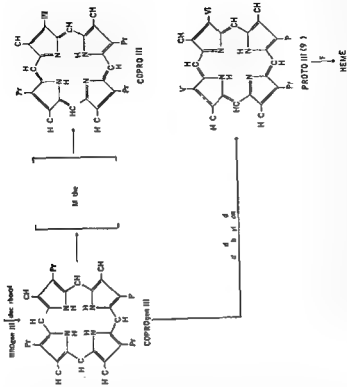
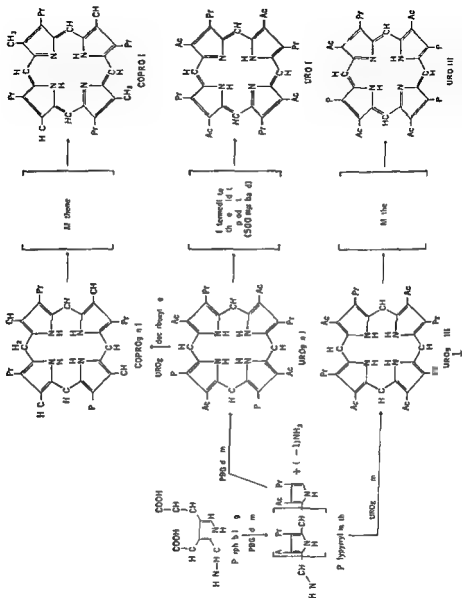


Fig 30-9 Scheme of heme biosynthesis from porphobilinogen. Ac —CH<sub>2</sub>COOH. Pr —CH<sub>2</sub>CH<sub>2</sub>COOH. Vi —CH=CH<sub>2</sub>. Urohydrogen COH R(OH) uroporphyrinogen coproporphyrinogen URO COH RO 1 ROTO uroporphyrin coproporphyrin protoporphyrin (By per version of S G anick and D Mauzerall [110,1])

level intermediate between uroporphyrinogen and uroporphyrin [118 114 116] Mauzerall and Granick have suggested that these intermediates may be porphomethene and porphodimethene both of which show strong absorption at 500 mμ [118] The former has two and the latter four hydrogen atoms less than uroporphyrinogen (Fig 30-10) These partially oxidized intermediates may be obtained by slow oxidation of uroporphyrinogen with iodine in the dark or by reduction of uroporphyrin with sodium amalgam [118 119] Enzymatic oxidation of uroporphyrinogen catalyzed by a heat labile oxidase obtained from spinach leaves [116] appears to proceed along similar lines The oxidase can be separated from PBG deaminase by starch block electrophoresis and is markedly inhibited by hydroxylamine [116] Its specificity is not known and it appears to be of little biologic significance The same holds true for non enzymatic oxidation of uroporphyrinogen Since the amount of uroporphyrin normally excreted in the urine is exceedingly small as compared with the magnitude of heme synthesis the fraction of uroporphyrinogen that is oxidized and escapes from the biosynthetic path must be in



degree of specificity ALA PBG and uroporphyrin cannot replace uroporphyrinogen as substrates [113 118] Mercury copper manganese and oxygen strongly inhibit the reaction [113 118] the latter probably by enhancing autooxidation of the substrate to uroporphyrin On the other hand reduced glutathione and cysteine greatly increase the yield of coproporphyrinogen A heat stable ultrafiltrable cofactor has been described in *R. spheroides* [114] which appears to be essential for the enzymatic decarboxylation of uroporphyrinogen The isomer specificity of uroporphyrinogen decarboxylase is not known Both uroporphyrinogen I and III can serve as substrates [113 118] but the rate of decarboxylation appears to be higher for the type III isomer [113]

The removal of the four acetic acid side chains of uroporphyrinogen probably occurs stepwise and randomly so that intermediate porphyrinogens with seven six and five carboxyl groups are formed [104 113 120] Since the decarboxylating enzyme(s) appears to have a low Michaelis constant and a high turnover number [113] the concentration of the intermediate porphyrinogens at any one given time is very low and the reaction is rapidly carried through to coproporphyrinogen As for uroporphyrinogen photocatalytic autooxidation of porphyrinogens with less than eight carboxyl groups is probably of little biologic importance since exclusion of light and the presence of antioxidants in the cells maintain them in a reduced state Under aerobic conditions in vitro however porphyrins with seven to five carboxyl groups are readily obtained [103 104 113] It is not known whether the successive removal of carboxyl groups of uroporphyrinogen to form ultimately coproporphyrinogen is catalyzed by a single or by several closely related enzyme systems [113] For convenience the term uroporphyrinogen decarboxylase is used although the enzyme as defined can react with porphyrinogens which exhibit fewer carboxyl groups than uroporphyrinogen

With uroporphyrinogen I as substrate coproporphyrinogen I or its oxidation product coproporphyrin I is the end product since further decarboxylation of the type I isomer series does not occur [113 118 120 128] Protoporphyrin or heme corresponding to type I isomer has never been demonstrated in nature [121 128] With uroporphyrinogen III on the other hand enzymatic decarboxylation may yield porphyrins of type III isomer with less than four carboxyl groups [118 129] Granick and Mauzerall [129] have presented evidence which strongly suggests that an enzyme bound to insoluble components of chicken erythrocytes or of *Fuglena* is involved in the oxidative decarboxylation of coproporphyrinogen III to form the dicarboxylic protoporphyrinogen III The latter through intermediate stages of autooxidation may be converted to protoporphyrin III (9a) [104 129] The requirement for a particulate enzyme system for oxidative decarboxylation of coproporphyrinogen III has also been demonstrated by the findings of Rimington and Booy



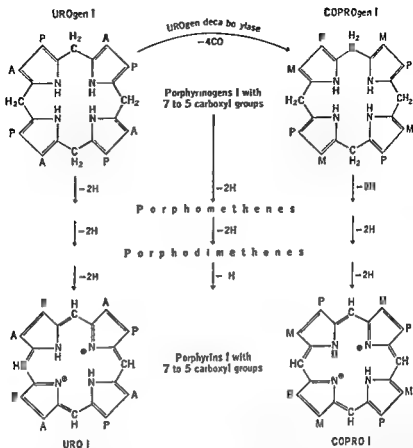


Fig 30-10 Stepwise conversion of uroporphyrinogen to coproporphyrinogen by uroporphyrinogen decarboxylase with formation of intermediate porphyrinogens with 7  $\blacksquare$  and  $\blacksquare$  carboxyl groups. Each porphyrinogen can undergo stepwise oxidation to its respective porphomethene, porphodimethene, and finally porphyrin. (For abbreviations see Fig 30-8.)

significant. It appears likely that the exclusion of light and the presence in the cells of antioxidants such as glutathione and cysteine are of importance in keeping most of the tetrapyrroles in a reduced state [113].

The major fraction of the uroporphyrinogen is enzymatically decarboxylated to porphyrinogens with less than eight carboxyl groups [103, 113, 114, 118, 124] (Fig 30-10). Uroporphyrinogen decarboxylase activity has been demonstrated in erythrocytes of man [124, 125], ducks [112], and chickens [127], in rabbit reticulocytes [113], in *Chlorella* [109], and in *Rhodospseudomonas spheroides* [114]. With enzyme preparations obtained from rabbit reticulocytes [113] or *Chlorella* [118], anaerobic incubation of uroporphyrinogen resulted in formation of coproporphyrinogen exhibiting four carboxyl groups. The enzyme has a high

of succinyl CoA pyridoxal phosphate for the condensation of succinyl CoA with glycine and copper as part of ALA dehydratase. It appears likely that other cofactors may also be required but their nature has not yet been recognized [114].

4 ALA and PBG are obligatory intermediates in the biosynthesis of porphyrins and of heme.

5 Porphyrinogens and porphyrins of the type I isomer series are by products of heme synthesis without known physiologic function. In vivo the biosynthetic pathway is conditioned in such a way that formation of porphyrins of type I isomer is insignificant as compared with the amount of heme formed.

6 With the probable exception of protoporphyrin III (9a) porphyrins of the type III isomer series are not intermediates in heme biosynthesis but are by products resulting from irreversible oxidation of the respective porphyrinogens. The true intermediates are porphyrinogens of the type III isomer which are reduced porphyrins containing an additional six hydrogen atoms. Conversion of porphyrinogens to porphyrins occurs by photocatalytic autoxidation or by the enzyme porphyrinogen oxidase. In vivo oxidation of porphyrinogens to porphyrins is minimized by the exclusion of light and by the presence of antioxidants.

7 The dynamics of the uroporphyrinogen decarboxylase system favor the formation of porphyrins with eight carboxyl groups (uroporphyrin) and with four carboxyl groups (coproporphyrin) but smaller amounts of porphyrins with seven, six, five and three carboxyl groups are also formed.

## METABOLISM OF PORPHYRINS AND PORPHYRIN PRECURSORS IN THE INTACT ORGANISM

The bone marrow of an adult individual forms approximately 300 mg heme per day. This amount is needed for hemoglobin formation to compensate for the physiologic decay of aged erythrocytes [130]. Additional heme is synthesized for the formation of other heme proteins but the lack of precise information regarding the rate of turnover of these compounds precludes exact estimation of the total daily heme production. Undoubtedly it is considerably larger than the 300 mg required for hemoglobin synthesis in the bone marrow since heme containing compounds which are believed to be synthesized *in loco* are present in almost all aerobic cells [60]. Relative to this rate of heme formation the excretion of porphyrins and of porphyrin precursors in urine and bile is very small. If one visualizes the excreted compounds as the sum of all porphyrins and porphyrin precursors which escape during heme formation it becomes obvious that heme biosynthesis is proceeding with a remarkable degree of efficiency. It is not known how much individual organs contribute to the

[130] that with ALA or PBG as substrates addition of mitochondria to a system consisting of hemolyzed human erythrocytes greatly enhances the formation of protoporphyrin III (9a). Conversion of coproporphyrinogen III to protoporphyrin III (9a) not only involves decarboxylation of the two propionic acid side chains but it requires formation of two vinyl groups. It is therefore not surprising that *in vitro* biosynthesis of protoporphyrin III (9a) from ALA [131] PBG [108] uroporphyrinogen III [118] or coproporphyrinogen III [120] requires either aerobic conditions or the presence of some electron acceptor.

Protoporphyrin III (9a) is used for the formation of heme. It is not known whether iron is inserted at an intermediate stage of autooxidation of coproporphyrinogen or into protoporphyrin itself. The latter seems more likely since it is well known that ferrous iron readily coordinates with porphyrins but not with porphyrinogens [113-132]. *In vitro* heme may be formed nonenzymatically by simple incubation of protoporphyrin with ferrous iron at 37°C under nitrogen [132]. On the other hand Krueger et al. [133] and Goldberg [134] have presented evidence which suggests that *in vivo* incorporation of iron may be catalyzed by an enzyme system present in nucleated erythrocytes.

#### RECAPITULATION

The nearly complete elucidation of the pathways by which glycine and succinate are used for the biosynthesis of porphyrins and of heme provides an impressive example of the biochemical progress made during the last decade. There can be little doubt that the few remaining regions of uncertainty and of ignorance will soon be explored and clarified. This applies particularly to the enzymatic mechanism which catalyzes the condensation of 4 moles of PBG to form the asymmetric uroporphyrinogen III and also to the steps involved in the oxidative decarboxylation of coproporphyrinogen III to protoporphyrin III (9a).

Some aspects of the biosynthetic pathway which in the intact organism are believed to be of particular importance with regard to porphyrin metabolism are summarized below.

1 While most of the individual biosynthetic steps proceed in the absence of oxygen two reactions require aerobic conditions: (a) the formation of succinyl coenzyme A which is dependent on a functioning tricarboxylic acid cycle; (b) the oxidative decarboxylation of coproporphyrinogen III to protoporphyrin III (9a).

2 These two reactions are catalyzed by enzyme systems which are bound to cell particles and which appear to be absent in mature non-nucleated erythrocytes. On the other hand the steps from succinyl CoA to coproporphyrinogen III are catalyzed by soluble enzymes present both in nucleated cells and in mature mammalian erythrocytes.

3 The following cofactors are essential: pantothenate for the formation

## PORPHYRINS

Normal human urine contains small amounts of porphyrins. The predominant one is coproporphyrin which is excreted at a daily rate of 100 to 300  $\mu\text{g}$  [148] the values being somewhat smaller in the female [149]. Both types I and III isomers are present but the ratio is subject to considerable variation [151-152]. In a variety of conditions such as hemolytic anemia, liver disease, and lead poisoning a slight to moderate increase in urinary coproporphyrin excretion is observed [148] and significant changes in isomer ratio have been reported [18]. Coproporphyrin is also present in the bile where the daily excretion rate is estimated at 400 to 1000  $\mu\text{g}$  [148]. Thus elevated values for urinary coproporphyrin excretion may result not only from increased porphyrin production but also from liver damage leading to diversion of excretion from the biliary to the urinary route [18]. For example, Hoffbauer and his coworkers [153] have found that in rats injected coproporphyrin III is entirely eliminated by the bile unless the bile ducts are occluded or the liver damaged by carbon tetrachloride.

Other porphyrins present in normal human urine include uroporphyrin [148-154] and traces of porphyrins with seven, six, five, and three carboxyl groups [151-155-156]. Most of the uroporphyrin appears to be of type I isomer with only trace amounts of uroporphyrin III [154-157].

Protoporphyrin is normally absent from the urine [18-148] but is present in bile and feces [18-158]. Even in Sedormid poisoning where very large amounts of protoporphyrin are excreted via the bile this porphyrin could not be demonstrated in the urine [34]. As a general rule it can be said that ALA, 1-BG, and uroporphyrin are mainly excreted in the urine while coproporphyrin is preferentially and protoporphyrin exclusively eliminated via the bile.

A large fraction of the porphyrins present in normal urine is excreted in the form of *precursors* which are converted to porphyrins by exposure to light and air or more efficiently by oxidation with dilute iodine [159-161]. These precursors include probably porphyrinogens 1-BG, and possibly ALA, all of which can be converted nonenzymatically to porphyrins [100-144-162]. It appears likely that the quantity and isomer type of porphyrins formed from precursors in the urine depends to some extent on external factors such as pH of the urine and type of oxidizing agent employed [122].

Except for circulating erythrocytes, information regarding the concentration of free porphyrins in human tissues is scant. Erythrocytes contain approximately 1 to 60  $\mu\text{g}$  protoporphyrin and 1 to 2  $\mu\text{g}$  coproporphyrin per 100 ml of cells [149-163]. These values may be greatly increased in iron deficiency, hemolytic anemia, lead poisoning, and other disorders of erythropoiesis [19-39-148-164].

'pool' of porphyrins and porphyrin precursors that are excreted but it appears likely that the bone marrow and the liver are major contributors

### $\delta$ AMINOLEVULINIC ACID AND PORHOBINOCEN

In man approximately 2 mg ALA is excreted in the urine per day [19 37 136 137]. No data are available for its excretion in bile but it is believed to be negligible [32 133]. Significant elevation of ALA excretion in the urine is observed in lead poisoning [139]. On the other hand Sedormid poisoning in rabbits does not appear to result in increased ALA excretion [37] although the concentration of PBG in the urine is high [36].

Injection or oral administration of labeled ALA to man and to animals results in the excretion of a large portion of the unaltered compound in the urine [32 138 140 141]. Smaller fractions are converted to PBG, to protoporphyrin and to stercobilin the former being excreted in the urine the latter two in the bile [32, 138 140]. All three compounds are believed to be formed outside the hematopoietic system, possibly in the liver [133, 140]. In addition ALA- $14\text{C}^{14}$  gives rise to labeled  $\text{CO}_2$  in the breath [32]. A small fraction of the injected ALA is used for heme biosynthesis in immature red blood cells [70 82 138 140] but in contrast to the observations *in vitro* incorporation of ALA into heme of circulating erythrocytes is several times less than that found with equivalent amounts of labeled glycine [32 138 140]. This may in part be explained by the rapid renal excretion of the injected ALA either in unchanged form or after its conversion to PBG. In addition penetration of ALA into immature red blood cells may be a rate-limiting factor [32 139 140].

In healthy individuals daily excretion of PBG in the urine is approximately 1.0 to 1.5 mg [19 37 136 137]. At the low concentrations which exist in normal urine the conventional test for detection [142] is inadequate and gives negative results [143]. Increased urinary excretion of PBG at times is observed in patients with hepatic malignant or infectious diseases [144] and is frequently present in lead poisoning [139 147]. In animals poisoned with Sedormid [36] or related compounds [37 145] large amounts of PBG are excreted in the urine and are present in the liver but not in the bone marrow or circulating erythrocytes [36].

If injected parenterally PBG is rapidly excreted in the urine [146], fecal excretion or conversion of PBC to porphyrins is insignificant [141, 146]. In animals injected PBG cannot be demonstrated in the liver [146] suggesting that it may not pass the liver cell membrane. Similar findings have been reported for fowl erythrocytes *in vitro* [96]. It is evident however that the impermeability of the cell membrane is only relative since in instances where endogenous PBG production is increased it does escape from the cells and gain access to the urine. Thus in acute porphyria of man and in Sedormid porphyria of animals large amounts of PBG are derived from the liver and excreted in the urine [36 41 146].

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Except for circulating erythrocytes information regarding the concentration of free porphyrins in human tissues is scant. Erythrocytes contain approximately 15 to 60  $\mu\text{g}$  protoporphyrin and 1 to 2  $\mu\text{g}$  coproporphyrin per 100 ml of cells [148-163]. These values may be greatly increased in iron deficiency, hemolytic anemia, lead poisoning and other disorders of erythropoiesis [18-39-148-164].

## PHARMACOLOGIC ACTION OF PORPHYRINS AND OF PORPHYRIN PRECURSORS

### PHOTOSENSITIVITY

The photosensitizing activity of porphyrins is well known and has been the subject of many studies [22 165-169]. This property is probably related to the intense fluorescence of porphyrins [22] since both are produced most effectively by long wave ultraviolet radiation of about 400 mμ wavelength corresponding to the peak absorption of porphyrins in the ultraviolet region of the spectrum [22 170-172]. Uroporphyrin is believed to exhibit the strongest sensitizing effect followed by coproporphyrin, protoporphyrin has few if any photosensitizing properties [22 167 172 173]. In the famous self-experiment of Meyer Betz [168] 200 mg hematoporphyrin was injected intravenously this resulted in marked erythema and edema of the exposed parts of the body. Similar observations have recently been reported by Schwartz [174]. The photodynamic effect in the skin may be mediated by release of histamine [175].

In spite of the recognized relation between porphyrins and photosensitivity [168] the pathogenesis of *hydra aestivale* the vesicular eruption occurring on exposed parts of patients who suffer from photosensitive porphyria is not fully understood. Blum and his coworker [170] failed to produce the characteristic vesicles by exposing areas of skin of a patient with erythropoietic porphyria to ultraviolet light although the wavelength of the emitted light corresponded to the absorption maximum of porphyrins. Similar negative results were reported by Watson and by Gray [22 176]. It appears likely that only continued and chronic exposure to sunlight results in formation of vesicles. This is in line with the observation made in some of the patients with photosensitive porphyria that *hydra aestivale* may not become manifest until midsummer but that the vesicular lesions then persist well into the fall [177].

Administration of ALA to human brings results in marked but transient hypersensitivity to sunlight [82 140]. In rats injection of ALA followed by exposure to radiation from a carbon arc lamp produces marked photosensitivity [178]. Injection of PBG has no such effect. This difference is believed to be related in part to the rate at which these compounds can enter epidermal cells [178]. Fluorescence microscopic examination of skin obtained from animals injected with ALA suggests that ultraviolet radiation may enhance the intracellular conversion of ALA to uroporphyrin [173]. This is reminiscent of the photocatalytic autooxidation of porphyrinogens to porphyrins *in vitro* a process which is sensitized by the product porphyrin [105]. *In vivo* de Mello [179 180] observed that in rabbits poisoned with lead acetate or injected with the photosensitizing dye rose bengal ultraviolet irradiation results in a

marked increase in urinary coproporphyrin excretion. In analogy with the observations *in vitro* it is tempting to attribute this extra excretion of pigment to photocatalytic conversion of precursors to porphyrins [181]. It is not clear, however, why the majority of patients with acute porphyria are free from photo-sensitivity, although large amounts of porphyrin precursors are present in the liver [41] and are excreted in the urine.

The above findings may be summarized as follows. Increased concentration of preformed porphyrins in the body undoubtedly predisposes to cutaneous photosensitivity. Light of near ultraviolet wavelength is particularly effective. Vesicular eruptions of the skin are produced only after prolonged and chronic exposure to sunlight. Under experimental conditions irradiation with ultraviolet light appears to promote conversion of porphyrin precursors to porphyrins, resulting in photo-sensitivity and increased porphyrin excretion in the urine. These observations suggest that photo-sensitivity is a *relative* phenomenon and may depend on such factors as light source, length and intensity of exposure, types of porphyrins and of precursors present, and their relative concentration in the skin and in the extracellular fluid.

#### OTHER PHARMACOLOGIC EFFECTS

In addition to their photosensitizing properties, porphyrins are said to produce a variety of toxic effects, including vascular and intestinal spasm and neuropathologic changes [47, 182, 183]. Reinvestigations of the pharmacologic action of naturally occurring porphyrins, however, have failed to produce convincing evidence which would suggest demonstrable effects of these compounds on smooth muscles or on the nervous system [178, 184-186]. Further, neither injection of hematoporphyrin [168] nor excessive production of endogenous uroporphyrin, as in congenital erythropoietic porphyria, gives rise to intestinal or neurologic symptoms. Nor are such symptoms produced by injection of PBG or ALA in man or in animals [82, 140, 178, 184, 185, 187]. *In vitro* these compounds were found to be without effect on smooth muscle of rat intestine [178, 184].

In view of the negative findings, it appears doubtful whether the intestinal, vascular, and neurologic manifestations of acute porphyria can be ascribed to direct toxic effects of porphyrins or of porphyrin precursors [121, 178, 181].

#### CONGENITAL ERYTHROPOIETIC (PHOTOSENSITIVE) PORPHYRIA

##### INCIDENCE AND CLINICAL MANIFESTATIONS

Erythropoietic porphyria is a rare congenital disease occurring much less frequently than other forms of porphyria. The prominence of photo-



sensitivity and of massive porphyrinuria has often caused it to be confused with the hepatic types of cutaneous porphyria [13], although the pathogenesis of the latter disorders is fundamentally different [41]. In the earlier literature [13, 188] many of the reported cases clearly had features of the hepatic forms while in other instances the data were insufficient to permit distinction [15]. A reexamination of the cases of so-called congenital photosensitive porphyria reported up to 1964 permitted the diagnosis of erythropoietic porphyria beyond reasonable doubt in a total of 34 cases [15]. An additional 7 cases have since come to the author's attention. The 41 cases and some of their essential features are summarized in Table 30-1. Twenty-four of the patients were female and 17 male. In the earlier literature it was generally held that congenital porphyria was more common in males but this was undoubtedly caused by confusion with porphyria cutanea tarda which is more frequent in males [35-40]. As seen in Table 30-1 the disease has a wide racial distribution and has been observed in children of Japanese, Bantu, and Sudanese extraction [190, 199, 202, 219, 220, 236].

The first sign suggesting the presence of erythropoietic porphyria is usually the excretion of red urine containing much uroporphyrin but no PBG. This may be noted at birth or only during the first years of life. Although porphyrinuria is probably present at all times, the amounts of porphyrins excreted and hence the red color of the urine may show considerable daily or seasonal fluctuation [8, 41, 203]. In some instances the color of the urine was light except during periods of active photodermatitis occurring during the summer months [8, 41, 203]. In others large and relatively constant amounts of uroporphyrin were excreted over long periods of time [19].

Photosensitivity is frequently absent in the neonatal period but it may become apparent during the first years of life as exposure to sunlight increases. A vesicular or bullous eruption appears on the face, the back of the hands, and other exposed parts of the body. This is commonly referred to as *hydra aestivale*, the adjective indicating the seasonal recurrence of the lesions. The vesicles contain a serous fluid which may exhibit red fluorescence, heal slowly, and leave depressed pigmented scars. Infected bullae often ulcerate, causing marked scarring and deformity involving particularly the tips of the fingers, the ears, nose, and eyelids. After years of repeated attacks, severe mutilation may ensue with contractions of the face and loss of parts of digits and ears [13]. In other cases the cutaneous manifestations may be relatively mild, resulting in little if any scarring. In several patients a definite decrease in photosensitivity and a reduction in urinary porphyrin excretion followed splenectomy [41, 203, 207]. In one of these children [203] removal of the spleen resulted in virtual loss of photosensitivity, but porphyrin-containing vesicles were observed on those parts of the hands which became infested with scabies [177].

Hypertrichosis is a frequent finding in these patients [13 22] Fine blond downy hair resembling lanugo may cover the face and extremities [13 22 190 204 214] Deciduous and permanent teeth may show a red or brownish discoloration (erythrodontia) [13 202] but in some cases this has been rather inconspicuous [18] Under ultraviolet light however the teeth always exhibit marked red fluorescence Deposition of porphyrin in the developing teeth and in bones is believed to be due to its physical affinity for calcium phosphate [18] The presence of porphyrin in the deciduous teeth may indicate that the metabolic disorder was already present during fetal life [191]

Splenomegaly is an almost constant feature of the disease (Table 30 1) It may be absent in the neonatal period and be detected only as the patient grows older [15] In one patient the spleen was enlarged in early childhood but could no longer be palpated 10 years later [192] In three patients splenomegaly was absent this was correlated with normal erythrocyte survival and lack of hemolytic anemia [217 225] Hypertension and abdominal or neurologic symptoms so frequently observed in other forms of porphyria are not found in erythropoietic porphyria

### HEMATOLOGIC FINDINGS

In the majority of the reported cases increased hemolytic activity was present as indicated by the following findings normochromic anemia associated with elevated reticulocyte levels and circulating normoblasts normoblastic hyperplasia of the bone marrow increased excretion of fecal urobilinogen [16] Rarely the anemia was severe leading to early death [195] or requiring multiple transfusions [227] but in most patients the reduction in hemoglobin concentration was only slight and the hemolysis was largely compensated by increased red cell production It was stated that the famous case Petry [202] at the time of his death suffered from pernicious anemia in addition to congenital porphyria but Aldrich et al [203] raised justified doubt as to the correctness of this interpretation of the autopsy findings and suggested that Petry may have had an unrecognized hemolytic anemia with splenomegaly [15] In another patient originally reported by Ashby [196] and later by Carrod [197] outspoken hemolytic anemia was repeatedly demonstrated [197] At the time of her death at age 27 she was found to have a coarse-nodular cirrhosis of the liver and the bone marrow showed extensive myeloid hyperplasia and no significant erythroid haemopoiesis [197] The available information is inadequate to permit speculation as to the possible factors which could have led to this marrow failure [19]

Direct estimation of erythrocyte life span has been performed in seven patients [210 212 214 217 228 229 230] five of whom had hematologic findings suggestive of hemolytic anemia A decreased survival of autologous erythrocytes was demonstrated by the glycine- $^{51}\text{Cr}$  method in the two patients studied by Grinstein et al [228] and Watson et al [212]

TABLE 301 CASES OF CONGENITAL ГИПЕРОБИЧНО ПОРФИРИЯ REPORTED IN THE LITERATURE UP TO 1958

| Sex and age         | Y    | Etiology and symptoms |           |          |             |           | Remarks   |
|---------------------|------|-----------------------|-----------|----------|-------------|-----------|---|
|                     |      | Symptoms              | Pathology | Autopsy  | Diagnosis   | Prognosis |   |
| And. a [60]         | 1898 | M. Eng. h.            |           | 4        | 0           | 0         | Two brothers, both affected with erythema.      |
| Ad. [9]             | 1898 | M. Eng. h.            |           | 3        | 0           | 0         | Time of appearance of erythema not established. |
| Ad. h. [189]        | 1911 | F. h. i. n.           |           | 14( )    | Hypoc.      | 0         | Age 10 years in 1932 [189]                      |
| C. b. h. [190]      | 1914 | M. h. i.              |           | 3        | + type test | 0         |   |
| M. h. y. [191 192]  | 1922 | M. F. h. h.           |           | B. r. h. | Hem. lytic  | +         |   |
| M. h. y. [191]      | 1922 | M. F. h. h.           |           | 2        | Hem. lytic  | +         |   |
| C. y. [192 194]     | 1924 | F. Eng. h.            |           | 5        | Hem. lytic  | +         |   |
| q. t. [195]         | 1926 | M. J. d.              |           | B. r. h. | Hypoc.      | +         |   |
| As. h. y. [192 196] | 1926 | F. J. d. b.           |           | B. r. h. | Hem. lytic  | +         |   |
| S. b. m. d. [198]   | 1926 | P. C. m. a.           |           | 2        | Hem. lytic  | +         |   |
| X. t. g. w. [199]   | 1927 | F. J. p. a. e.        |           | B. h.    | Hem. lytic  | +         |   |
| M. a. k. [200]      | 1928 | M. J. d. a. e.        |           | 18( )    | Hem. lytic  | +         |   |
| M. t. o. k. [200]   | 1928 | F. J. p. a. e.        |           | 3        | Hem. lytic  | +         |   |
| M. [201]            | 1929 | M. h. i. t.           |           | B. h.    | + type test | +         |   |
| B. w. [202]         | 1929 | M. G. n.              |           | B. h.    |             | +         |   |
| M. r. [203]         | 1931 | M. J. a. l. a.        |           | 5(7)     | Hem. lytic  | +         |   |
| T. a. g. [171 204]  | 1933 | M. W. h. o.           |           | 1        | + type test | +         |   |
| D. e. M. [197]      | 1934 | F. A. g. t.           |           | 5        | H. lytic    | 0         |   |
| H. e. d. [205]      | 1938 | F. S. p. h.           |           | 1        | 0           | 0         |   |
| H. e. n. d. [205]   | 1938 | F. S. p. h.           |           | 1        | 0           | 0         |   |
| H. d. [205]         | 1938 | F. S. p. h.           |           | 1        | 0           | 0         |   |
| H. e. r. d. [206]   | 1938 | M. S. p. h.           |           | 1        | 0           | 0         |   |
| H. d. [206]         | 1938 | M. S. p. h.           |           | 1        | 0           | 0         |   |

TABLE 30.1. CLERS OF CO. CENTRAL TRYPTHOGENIC PORCINIA REPORTED IN THE LITERATURE LP TO 1958 (Continued)

| Y    | C no th     | E self i fput i |      |                  |               |   | A mms | Spd on only | Ergth o d too                     | R m k |
|------|-------------|-----------------|------|------------------|---------------|---|-------|-------------|-----------------------------------|-------|
|      |             | q               | g    | ag ad i fput m b | g             | g |       |             |                                   |       |
| 1928 | P b y [200] | F               | Wh t | B th             | H m lyt       | + | +     | +           | L t tuded by Lo d West of m o R t |       |
| 1938 | D t r [201] | F               | Wh t | B                | + typ t t ted | + | +     | +           | t ober g l i (1)                  |       |
| 1947 | D b y [202] | F               | Wh t | B th             | sed b m ly    | + | +     | +           | epi tom d 1957 th m n R t         |       |
| 1948 | M y [203]   | M               | F b  | F 12             | ~             | + | -     | +           | h m lyt m [204]                   |       |
| 1949 | M y [204]   | F               | F b  | F 12             | ~             | + | -     | +           | epi t may 1913 21(1)              |       |
| 1949 | M y [205]   | M               | F b  | F 12             | H m lyt       | + | +     | +           | N mal e f f ch m t d yth y t      |       |
| 1949 | C t t [206] | M               | H t  | 3 6              | + much        | 0 | 0     | 0           | [207]                             |       |
| 1950 | P b y [207] | F               | B t  | 11 (m)           | H lyt         | + | +     | +           | B th f abo t aa                   |       |
| 1951 | M b y [208] | F               | Wh t | 3                | H m lyt       | + | +     | +           | lit thy bl e                      |       |
| 1952 | P b y [209] | F               | Wh t | Do g b t         | H lyt         | + | +     | +           | Par t f t                         |       |
| 1953 | P b y [210] | F               | H t  | B th             | H m lyt       | + | +     | +           | epi tom y th m f ph tose m        |       |
| 1954 | P b y [211] | F               | F b  | 3                | H m lyt       | + | +     | +           | t ly t may th p t l m m f ph to   |       |
| 1954 | P b y [212] | F               | F b  | 1                | H m lyt       | + | +     | +           | epi t may th p t l m m f ph to    |       |
| 1954 | P b y [213] | F               | F b  | 1                | H m lyt       | + | +     | +           | epi t may th p t l m m f ph to    |       |
| 1954 | P b y [214] | F               | F b  | 1                | H m lyt       | + | +     | +           | epi t may th p t l m m f ph to    |       |
| 1954 | P b y [215] | F               | F b  | 1                | H m lyt       | + | +     | +           | epi t may th p t l m m f ph to    |       |
| 1954 | P b y [216] | F               | F b  | 1                | H m lyt       | + | +     | +           | epi t may th p t l m m f ph to    |       |
| 1954 | P b y [217] | F               | F b  | 1                | H m lyt       | + | +     | +           | epi t may th p t l m m f ph to    |       |
| 1954 | P b y [218] | F               | F b  | 1                | H m lyt       | + | +     | +           | epi t may th p t l m m f ph to    |       |
| 1954 | P b y [219] | F               | F b  | 1                | H m lyt       | + | +     | +           | epi t may th p t l m m f ph to    |       |
| 1954 | P b y [220] | F               | F b  | 1                | H m lyt       | + | +     | +           | epi t may th p t l m m f ph to    |       |
| 1954 | P b y [221] | F               | F b  | 1                | H m lyt       | + | +     | +           | epi t may th p t l m m f ph to    |       |
| 1954 | P b y [222] | F               | F b  | 1                | H m lyt       | + | +     | +           | epi t may th p t l m m f ph to    |       |
| 1954 | P b y [223] | F               | F b  | 1                | H m lyt       | + | +     | +           | epi t may th p t l m m f ph to    |       |
| 1954 | P b y [224] | F               | F b  | 1                | H m lyt       | + | +     | +           | epi t may th p t l m m f ph to    |       |
| 1954 | P b y [225] | F               | F b  | 1                | H m lyt       | + | +     | +           | epi t may th p t l m m f ph to    |       |
| 1954 | P b y [226] | F               | F b  | 1                | H m lyt       | + | +     | +           | epi t may th p t l m m f ph to    |       |
| 1954 | P b y [227] | F               | F b  | 1                | H m lyt       | + | +     | +           | epi t may th p t l m m f ph to    |       |

Kx + -pr sent - = best O = t m t  
N ye See al o Add d m t l i + j t ab t m

TABLE 30.1 CASES OF CONGENITAL FETTERLOCKED FOREBRAIN REPORTED IN THE LITERATURE UP TO 1958

| Senior author      | Year | K. cerebral features |           |              |                  |           | Pathological findings | Clinical course                        |
|--------------------|------|----------------------|-----------|--------------|------------------|-----------|-----------------------|--|
|                    |      | Sx                   | R. side   | Age at onset | 4 m              | Spl. side |                       |  |
| And m (7)          | 1898 | M                    | F gl h    | 4            | 0                | 0         | 0                     | Tw broth m t r h e d t h e d e d w t h |
| A d (9)            | 1898 | N                    | E gl h    | 14 ( )       | 0                | 0         | 0                     | f r a y m p t o m s                    |
| Rad h (189)        | 1911 | P                    | It l      | 14 ( )       | 0                | 0         | 0                     | Time of appearance of first sympt m of |
| C. pp. U (190)     | 1914 | M                    | It l n    | 3            | + type n t tated | +         | 0                     | l a d y a t t i                        |
|                    | 19   | N                    | E gl h    | B r h        | It m l y t e     | +         | 0                     | (f m e e t y d e d 195 in ren (195)    |
|                    | 19   | N                    | E gl h    | B r h        | H m l y t e      | +         | 0                     |  |
|                    | 1924 | F                    | E gl h    | 5            | H m o d y t e    | +         | 0                     |  |
|                    | 1906 | N                    | J t r e e | B r h        | H m o d y t e    | +         | 0                     |  |
| G. A. bby (1908)   | 1906 | P                    | E gl h    | B r h        | H m o d y t e    | +         | 0                     |  |
|                    | 1906 | F                    | G m a n   | 2            | H m l y t e      | +         | 0                     |  |
| H. m. dt (1908)    | 1927 | P                    | J p r e e | B r h        | H m l y t e      | +         | 0                     |  |
|                    | 1908 | N                    | J p r e e | 16 ( )       | H m l y t e      | +         | 0                     |  |
|                    | 1908 | N                    | J p r e e | 16 ( )       | H m o d y t e    | +         | 0                     |  |
| M. t. u. ka (1908) | 1908 | F                    | J p r e e | 3            | H m l y t e      | +         | 0                     |  |
|                    | 1908 | N                    | It a l n  | B r h        | + type n t tated | +         | 0                     |  |
|                    | 1908 | N                    | G m       | B r h        | +                | +         | 0                     |  |
| M. m (1908)        | 1931 | N                    | It a l    | 6 ( )        | H m l y t e      | +         | 0                     |  |
|                    | 1933 | N                    | Whit      | 1            | + type n t tated | +         | 0                     |  |
|                    | 1934 | P                    | A x t i   | 3            | H l y t          | +         | 0                     |  |
| T. u. s. g (1917)  | 1918 | P                    | q p n h   | 1            | 0                | 0         | 0                     |  |
|                    | 1938 | P                    | q p n h   | 1            | 0                | 0         | 0                     |  |
|                    | 1938 | P                    | q p n h   | 1            | 0                | 0         | 0                     |  |
| De. M. a. l (1917) | 1938 | P                    | q p n h   | 1            | 0                | 0         | 0                     |  |
|                    | 1938 | P                    | q p n h   | 1            | 0                | 0         | 0                     |  |
|                    | 1938 | P                    | q p n h   | 1            | 0                | 0         | 0                     |  |
| H. d (1908)        | 1938 | M                    | q p n h   | 1            | 0                | 0         | 0                     |  |
|                    | 1938 | M                    | q p n h   | 1            | 0                | 0         | 0                     |  |
|                    | 1938 | M                    | q p n h   | 1            | 0                | 0         | 0                     |  |
| H. m. d (1908)     | 1938 | M                    | q p n h   | 1            | 0                | 0         | 0                     |  |
|                    | 1938 | M                    | q p n h   | 1            | 0                | 0         | 0                     |  |
|                    | 1938 | M                    | q p n h   | 1            | 0                | 0         | 0                     |  |
| H. d (1908)        | 1938 | M                    | q p n h   | 1            | 0                | 0         | 0                     |  |
|                    | 1938 | M                    | q p n h   | 1            | 0                | 0         | 0                     |  |
|                    | 1938 | M                    | q p n h   | 1            | 0                | 0         | 0                     |  |

P. m. d.

Tw broth m t r h e d t h e d e d w t h  
f r a y m p t o m s  
Time of appearance of first sympt m of  
l a d y a t t i  
(f m e e t y d e d 195 in ren (195)  
ne l a t t i n g d e d w t h m l j m p t m  
D e d 1949 in t a t c o a f l l g d l r y  
(197)  
q p n h m l y t e d t u t t p e r f n e d  
q p n h f l l n a d c a s e  
H m o d y t e d j r e e d g a s t m f o a t o f f e t  
s y m p t o m s l l y a t t e d  
C a s e P r y e l e d t o l e p w  
b u t y h b e e n l m l y t e (1905)  
R e u l e d g a s i n 1948 (1905)  
q p n h w i t h p a r t i l r m a s f l l o b o  
a c c u s i t y  
T w o t r e } l y f o a s e t r e d u r l h y  
b i l l x } p e r t r i a l e

by Canivet et al [217] exhibited marked hemolytic anemia [215-216]. Furthermore between the two Sudanese siblings studied by Townsend, Coles and Barnes [220] one had marked hemolytic anemia while in the other hemoglobin concentration, erythrocyte count and reticulocyte count were normal although the bone marrow showed normoblastic hyperplasia.

The mechanism responsible for the hemolytic process is not understood. The absence of a positive Coombs test and the finding of decreased survival of porphyric cells in a normal recipient [214] may be suggestive of an intracorpuseular defect. On the other hand Hausmann's observation [232] that hematoporphyrin sensitizes normal red cells to hemolysis by light *in vitro* gave rise to the speculation that the presence of porphyrins in the circulating plasma may exert a hemolytic effect *in vivo* [229]. An alternative explanation is suggested by the observations made in the fluorescence microscope that only a fraction of the normoblasts and of the immature erythrocytes contains increased amounts of porphyrins [15]. If the presence of porphyrins predisposes erythrocytes to hemolysis it is conceivable that porphyrin-laden cells may have a shortened life span whereas cells which lack excessive amounts of porphyrin may survive normally. The presence of very high porphyrin concentrations in porphyric spleens [41-214] is compatible with this hypothesis. It is possible that the life span of porphyrin-containing cells may be further shortened by exposure of the patients to sunlight. It should be noted however that none of these explanations is based on concrete experimental evidence.

Studies of unstained bone marrow preparations in the fluorescence microscope show intense porphyrin fluorescence in nucleated red cells and to a lesser degree in reticulocytes [15-41-212] (Fig. 30-11). In normoblasts most of the porphyrin appears to be present either inside or at the surface of the cell nucleus (Fig. 30-11) [15]. A fraction of the normoblasts in all developmental stages and some of the reticulocytes in the peripheral blood fail to exhibit any red fluorescence [15]. There appears to be a clear separation between fluorescing and nonfluorescing cells. Furthermore fluorescing normoblasts exhibit a morphological abnormality in nuclear structure consisting of single or at times multiple nuclear inclusions (Fig. 30-12) which stain dark with benzidine (Fig. 30-13) and which show strong absorption in the 400 m $\mu$  band [15]. This nuclear abnormality is not present in normoblasts which lack porphyrin fluorescence. It is interesting to note that similar findings have been reported in congenital porphyria of cattle [212]. These observations suggest the possibility that two different lines of erythropoietic cells may be present and that only one exhibits the defect in porphyrin metabolism [15].

In patients in whom increased hemolysis was demonstrable the excre-

In the former patient splenectomy resulted in remarkable improvement in hemoglobin concentration porphyrin excretion, and photosensitivity [203] in the latter patient, the time which has elapsed since the operation is too short to permit adequate evaluation. In the case reported by London et al [210] the average life span of the erythrocytes appeared to be normal but the reticulocyte count was elevated and the marrow showed erythroid hyperplasia. In a study reported by Roenthal et al [214] erythrocytes of a patient with erythropoietic porphyria were infused into a recipient with ulcerative colitis, and the disappearance of the donor cells from the recipient's circulation was followed by differential agglutination. Although the analysis of the results was complicated by the fact that the recipient was bleeding the findings suggested a more rapid rate of removal of the porphyrin cells as compared to cells from a normal donor. Splenectomy in this patient resulted in only temporary improvement although 4 years postoperatively survival of chromated erythrocytes was almost normal [231]. On the other hand in two siblings studied by Carnet et al [217] red cell survival as estimated by the  $\text{Cr}^{51}$  method appeared to be normal and there was no evidence of hemolytic anemia as judged by normal hemoglobin concentration reticulocyte counts fecal urobilinogen excretion and absence of splenomegaly and of erythroid hyperplasia of the bone marrow.

These seemingly contradictory results may find a possible explanation in the observation that a hemolytic component may be present during one phase of the disease but absent at other times. Thus in a patient in whom over the course of 25 years hemolytic anemia had repeatedly been demonstrated [191-192] Cray et al [229-230] found that approximately half the erythrocytes appeared to have a mean life span of less than 20 days while other red cell fractions survived for periods of 40 to 70 days and for 110 to 120 days respectively. At the time of this study the patient was believed to have a hemolytic crisis as suggested by the presence of anemia reticulocytosis normoblasts in the peripheral blood and increased excretion of fecal urobilinogen. At a later date when the reticulocyte count had returned to normal a second study of erythrocyte survival suggested that at least 80 per cent of the cells appeared to have a normal life span while only a minor fraction appeared to be removed between the thirtieth and the eightieth day. All these values were calculated on the basis of data obtained by following the  $\text{N}^{15}$  content of circulating hemoglobin heme after administration of  $\text{N}^{15}$  labeled glycine (Fig. 30-2) [229-230]. The limitations of this method for determining the apparent life span of circulating erythrocytes must be kept in mind.

The above findings suggest that increased hemolysis though present in most of the cases may be intermittent in nature and at times may be so slight as to be difficult to detect. It is interesting that the porphyrin brother of the two patients with normal erythrocyte survival reported

by Canivet et al [217] exhibited marked hemolytic anemia [215-216]. Furthermore, between the two Sudanese siblings studied by Townsend, Coles and Barnes [220], one had marked hemolytic anemia while in the other hemoglobin concentration, erythrocyte count, and reticulocyte count were normal, although the bone marrow showed normoblastic hyperplasia.

The mechanism responsible for the hemolytic process is not understood. The absence of a positive Coombs test and the finding of decreased survival of porphyrin cells in a normal recipient [214] may be suggestive of an intracorporeal defect. On the other hand, Hausmann's observation [232] that hematoporphyrin sensitizes normal red cells to hemolysis by light *in vitro* gave rise to the speculation that the presence of porphyrins in the circulating plasma may exert a hemolytic effect *in vivo* [229]. An alternative explanation is suggested by the observations made in the fluorescence microscope that only a fraction of the normoblasts and of the immature erythrocytes contains increased amounts of porphyrins [16]. If the presence of porphyrins predisposes erythrocytes to hemolysis, it is conceivable that porphyrin-laden cells may have a shortened life span, whereas cells which lack excessive amounts of porphyrin may survive normally. The presence of very high porphyrin concentrations in porphyrin spleens [41-214] is compatible with this hypothesis. It is possible that the life span of porphyrin-containing cells may be further shortened by exposure of the patients to sunlight. It should be noted, however, that none of these explanations is based on concrete experimental evidence.

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In patients in whom increased hemolysis was demonstrable, the excre-



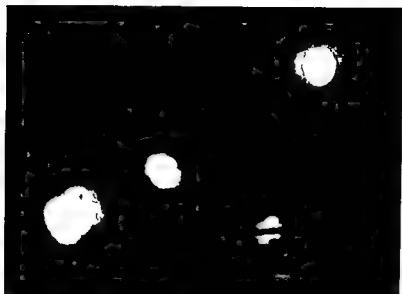


Fig 30-11 Unstained bone marrow smear of a patient with erythropoietic porphyria photographed in the fluorescence microscope ( $\times 800$ ). Red fluorescence (reproduced in white) is particularly intense in the normoblastic nuclei. The polychromatophilic erythrocyte in the left lower corner exhibits a lesser degree of porphyrin fluorescence. (By permission of J. Schmid et al. [41])



Fig 30-12 Bone marrow smear from a patient with erythropoietic porphyria stained with Wright's stain. The nucleus of the large normoblast shows a central inclusion. (By permission of J. Schmid et al. *Acta haematol* 10:153, 1953.)

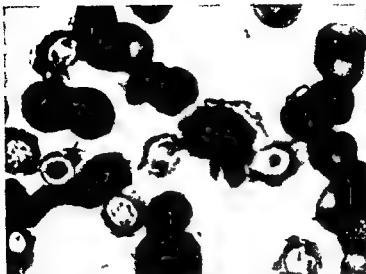


Fig 30-13 Bone marrow smear from a patient with erythropoietic porphyria stained with benzidine. Abnormal normoblasts contain dark-staining nuclear inclusions. Nuclei of normal immature normoblasts appear granular (left border); those of normal mature normoblasts white (left lower corner). Hemoglobin-containing erythrocytes stain dark (By permission of R. Schmid et al [16]).

tion of fecal urobilinogen was elevated [15 41 203 214 229]. More important was the observation that a major fraction of the excreted bile pigments was not derived from breakdown of hemoglobin of mature circulating erythrocytes [65 210 228 230]. While in normal subjects 80 to 90 per cent of the fecal urobilinogen originates from destruction of red cells which have reached the end of their physiologic life span [65 230 233] (Figs 30-2 30-14) this does not apply to erythropoietic porphyria [65 210 230]. At least 31 per cent and in some patients as much as 80 per cent of the excreted urobilinogen is derived from sources other than circulating hemoglobin (Fig 30-15). Most of this urobilinogen fraction is excreted during the first 20 days after the administration of labeled glycine (Fig 30-15). That it is not derived from destruction of circulating erythrocytes is demonstrated by the fact that there is no concomitant fall in labeled hemoglobin heme.

The source of this early labeled urobilinogen fraction is obscure. It appears unlikely that nonhemoglobin heme such as myoglobin and other heme proteins contribute significantly to its formation [210 230 234]. It has been suggested that it may be derived from developing red cells which are destroyed in the marrow or shortly after their release into the circulation or alternatively that heme which is formed in excess of globin may be discarded and converted to bile pigments [234 93]. As a third possibility it may be suggested that the increase in the

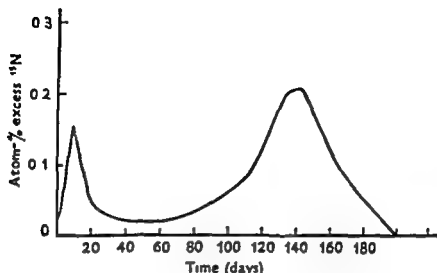


Fig. 30-14 Concentration of  $^{15}\text{N}$  in fecal sterco-bilin of a normal subject given 1 gm glycine- $^{15}\text{N}$  (31.65 atom per cent excess  $^{15}\text{N}$ ) during the first 4 days of the experiment (By permission of C. H. Gray [30])

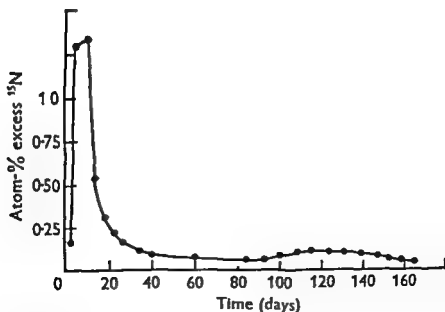


Fig. 30-15 Concentration of  $^{15}\text{N}$  in fecal sterco-bilin of a patient with erythropoietic porphyria given 1 gm glycine- $^{15}\text{N}$  (31.65 atom per cent excess  $^{15}\text{N}$ ) during the first 4 days of the experiment (By permission of C. H. Gray [30])

early labeled urobilinogen fraction may be related to the presence of two types of erythroid cells [15] as demonstrated in the fluorescence microscope. If it could be established that porphyrin containing erythrocytes have a much shorter life span than those which lack an increased pigment concentration, the observed pattern of bile pigment excretion would find a simple explanation. None of these hypotheses, however, is based on concrete experimental evidence.

#### CHEMICAL FINDINGS

The color of the urine of patients with erythropoietic porphyria may vary from a faint pink to a Burgundy red to a dark reddish brown depending upon the concentration of porphyrins. The amounts of uroporphyrin excreted are always increased and daily excretion of as much as 500 mg has been reported [13]. In addition, the urine contains large amounts of coproporphyrin, but the concentration is usually less than that of uroporphyrin [41, 203, 236]. Smaller amounts of porphyrins with seven, six, five, and three carboxyl groups have also been demonstrated [217, 227, 236]. Most of the urinary porphyrins are of the type I isomer [39, 63] but small fractions of uroporphyrin III have been identified [236, 237]. For coproporphyrin, the type I to type III isomer ratio may be as high as 99:1 [203] although small amounts of coproporphyrin III have been isolated in crystalline form [203].

PBG as identified by the conventional semiquantitative method [142] is consistently absent from the urine. Using more refined techniques, the daily excretion of PBG and of ALA in the urine is said to be within normal limits [227]. The feces regularly contain large amounts of coproporphyrin [13, 197, 203, 212, 220, 229] while the amount of uroporphyrin is much smaller [197, 203, 212]. Fecal excretion of protoporphyrin III (9a) though variable is usually not elevated. Most of the coproporphyrin is of type I isomer although small amounts of coproporphyrin III have been identified [197, 203].

Variable concentrations of uroporphyrin I and coproporphyrin I are regularly demonstrable in the plasma [41, 203, 212, 214]. Circulating erythrocytes contain high concentrations of uroporphyrin I [41, 203, 212, 214, 227] and somewhat lower concentrations of coproporphyrin I while for protoporphyrin III (9a) the values are usually not higher than those found in other hemolytic conditions [238]. Typical values in a patient studied before and after splenectomy are given in Table 30-2. Highest concentrations of these porphyrins are present in the bone marrow (Table 30-2) where the ratio of uroporphyrin I to coproporphyrin I is about the same as in the peripheral blood [41, 212, 227]. Most of the bone marrow porphyrins are present in developing erythroid cells (Fig. 30-11) [15, 203]. Uroporphyrin I in crystalline form has been isolated from bone marrow obtained by needle aspiration [41].

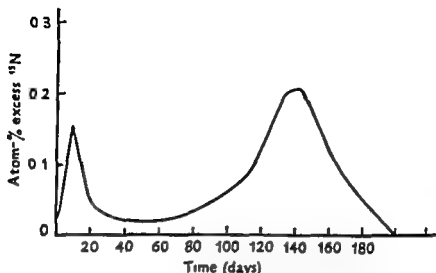


Fig. 30-14 Concentration of  $N^{15}$  in fecal sterolilin of a normal subject given 1 gm glycine- $N^{15}$  (31.65 atom per cent excess  $N^{15}$ ) during the first 4 days of the experiment (By permission of C. H. Gray [30])

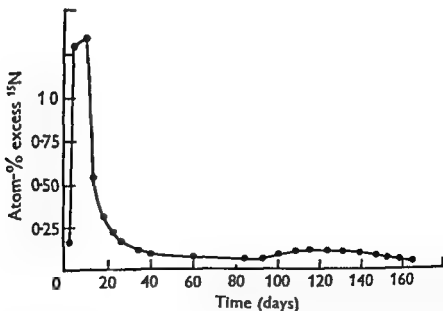


Fig. 30-15 Concentration of  $N^{15}$  in fecal sterobilin of a patient with erythropoietic porphyria given 12 gm glycine- $N^{15}$  (31.65 atom per cent excess  $N^{15}$ ) during the first 4 days of the experiment (By permission of C. H. Gray [30])

system which converts PBG to uroporphyrinogen III [114 124 125] Preheating of the system to 60 C however results in loss of the ability to form the type III isomer PBG being converted *only* to uroporphyrinogen I [124 125]

In erythrocytes of patients with erythropoietic porphyria Rimington and Booy [130] and MacRae [239] demonstrated that ALA and PBG are converted to porphyrinogens of *both* type I and type III isomers Thus red cells in erythropoietic porphyria resemble *normal* human erythrocytes in which the ability to form porphyrins of type III isomer has been partly destroyed It is obvious that in porphyric erythrocytes the metabolic defect is only a partial one since complete inability to synthesize porphyrins of type III isomer would preclude the formation of hemoglobin a situation which would be incompatible with life In formation as to the relative amounts of the two porphyrin isomers formed in porphyric cells is not yet available

It might be expected that in maturing porphyric red cells the highest rate of synthesis of uroporphyrinogen I would coincide with the phase of most active hemoglobin formation As Thorell [240] has shown this occurs in the later stages of the developing normoblast Part of the uroporphyrinogen I synthesized in these cells is oxidized to uroporphyrin I while another fraction is decarboxylated to porphyrinogens with less than eight carboxyl groups mainly to coproporphyrinogen I which in turn is converted to coproporphyrin I (Fig 30 9) The factors which determine the relative amounts of the e type I porphyrins formed in porphyric red cells are not known but it would appear that the amount of coproporphyrin I formed may depend in part on the rate at which uroporphyrinogen I is oxidized to uroporphyrin I Since this process is photocatalytic [105] and is sensitized by the product uroporphyrin it may provide a possible explanation for the observation that some of the patients with erythropoietic porphyria are found to excrete more uroporphyrin during the summertime when they are exposed to sunlight [8 41 203]

The mechanism by which porphyrins are released from the cells is not understood A simple calculation based on the concentration of porphyrins in the circulating red cells indicates that release of porphyrins from hemolyzed erythrocytes could not account for more than a relatively small fraction of the excreted pigments [41] The high porphyrin content of bone marrow suggests that a major fraction of the porphyrins present in plasma and excreted in urine and bile may be derived from developing red cells This is supported by the observation that the most intense porphyrin fluorescence is found in immature red cells which still have their nuclei [15] (Fig 30-11) The erythroid hyperplasia of the bone marrow resulting from the increased rate of hemolysis further augments the formation of type I porphyrins [39 41 212 227] The beneficial

Large amounts of uroporphyrin I are also present in the spleen [41 202 203, 212 214 227] On fluorescence microscopic examination fine dustlike granules of porphyrin are visible in the red pulp but not in the Malpighian corpuscles [41, 202] In contrast to the marrow, splenic cells

TABLE 30-2 PORPHYRIN CONCENTRATIONS IN ERYTHROCYTES BONE MARROW AND URINE OF A 6-YEAR-OLD GIRL WITH ERYTHROPOIETIC PORPHYRIA

| Date    | Erythrocytes $\mu\text{g}/100\text{ ml}$                   |       |       | Bone marrow $\mu\text{g}/100\text{ ml}$ |       |       | Urine $\mu\text{g}/24\text{ hr}$ |       |
|---------|--|-------|-------|---|-------|-------|----------------------------------|-------|
|         | URO  | COPRO | PROTO | URO                                     | COPRO | PROTO | URO                              | COPRO |
| 11/2/50 | 110  | 56    | 55    | 2635                                    | 302   | 121   | 8400                             | 2780  |
| 4/2/51  | 377  | 91    | 41    | 2432                                    | 614   | 180   | 21500                            | 3450  |
| 6/21/51 | 410  | 150   | 105   |   |       |       | 52300                            | 7800  |
| 6/21/51 | Splenectomy spleen contained 3400 $\mu\text{g}$ URO/100 gm |       |       |   |       |       |                                  |       |
| 7/3/51  |  |       |       |   |       |       | 1700                             | 400   |
| 10/6/51 | 192  | 20    | 60    | 176                                     | 81    | 281   | 690                              | 310   |

NOTE: URO (COPRO) PROTO = uroporphyrin coproporphyrin protoporphyrin respectively

SOURCE: R. Schmid et al. [41]

do not exhibit nuclear fluorescence [41] In the liver red fluorescence is minimal but considerable amounts of uroporphyrin I are demonstrable on extraction [41 202 212 214]

#### NATURE OF THE METABOLIC DEFECT

The findings discussed in the preceding sections indicate that in erythropoietic porphyria the primary defect probably involves overproduction of porphyrins of type I isomer for which the body has no use The defect appears to be confined to the hematopoietic system [15 41, 128 230] and may involve only part of the erythroid cells [15 202] Thus Fischer's original concept [59] that porphyrins of type I isomer are synthesized along with and parallel to hemoglobin formation appears to be essentially correct

As seen in Figs 30-8 30-9 the most likely site for the metabolic defect is in the enzymatic mechanism which converts PBG to uroporphyrinogen It may be recalled that PBG deaminase converts PBG only to uroporphyrinogen I whereas formation of uroporphyrinogen III requires the combined action of PBG deaminase and uroporphyrinogen isomerase It would appear that either overactivity of PBG deaminase or a defect in uroporphyrinogen isomerase would account for the increased formation of uroporphyrinogen I at the expense of the type III isomer [113 117] It has been noted that the latter enzyme is relatively heat labile [113, 114 117] Human red cell hemolysates contain the complete enzymatic

patients are homozygous for the trait. The sexes are equally affected. The lack of increased porphyrin excretion in parents and in normal siblings of patients [217-225] suggests that individuals who are heterozygous for the trait do not exhibit the metabolic abnormality. Thus the mode of inheritance of erythropoietic porphyria appears to be autosomal recessive.

#### CONGENITAL PORPHYRIA IN OTHER MAMMALS

**Cattle.** Congenital porphyria has been observed in cattle in South Africa, Denmark, England, and the United States [212, 244-247]. In all instances the disease was inherited as a simple Mendelian recessive characteristic. Heterozygous animals are clinically and biochemically normal [212, 246, 247]. Porphyric calves were obtained by breeding animals which were known to be heterozygous for the trait [212, 248].

Afflicted animals exhibit photosensitivity of those areas of the skin which are not pigmented or covered with dark hair [212, 248]. The first case of bovine porphyria recognized in Denmark was discovered because

the cow was not getting on at pasture but did well in the stable. When the animals are slaughtered, bones and teeth are found to be dark and to contain a large amount of porphyrin [212, 244, 246, 248]. The bovine form of congenital porphyria appears to have distinct similarities to the human disease. Anemia, reticulocytosis, and splenomegaly may be present [212], but splenectomy performed in one porphyric cow failed to result in significant improvement [212]. The spleen contained much porphyrin, mostly uroporphyrin I; the porphyrin concentration in the liver was insignificant [212, 247]. As in the human disease, highest porphyrin concentrations were found in the bone marrow [212], and on fluorescence microscopic examination unstained bone marrow preparations exhibited intense porphyrin fluorescence, which was most prominent in normoblastic nuclei. On staining, these cells exhibited abnormalities of nuclear structure similar to those described in human porphyria (Figs 30-11, 30-13). In contrast to what occurs in the human disorder, circulating erythrocytes contained relatively little uroporphyrin and coproporphyrin, but much protoporphyrin [212]. While most of the uroporphyrin and coproporphyrin in bone marrow and circulating erythrocytes was of type I isomer [212], the isomer type of the protoporphyrin has not yet been determined. It is conceivable that the high protoporphyrin content of bovine red cells may be due in part to formation of protoporphyrin I. If further studies should confirm this hypothesis, it would be the only instance in which protoporphyrin of type I isomer has been demonstrated in nature. It would further indicate that bovine erythrocytes must be capable of decarboxylating coproporphyrinogen I, an enzymatic mechanism lacking in red cells of the other species studied.



effect of splenectomy observed in some of the cases may be due to a reduction in the rate of hemolysis and hence in erythroid hyperplasia which results in reduced porphyrin formation [39]

### HEREDITARY CONSIDERATIONS

It was assumed by Carrod [241] and by Guenther [13] that erythropoietic porphyrin is an inborn error of metabolism probably inherited as a Mendelian recessive character. The data presently available support this belief but they do not bear out the additional claim that the condition is more common in the male [13, 242]. In fact of the 41 cases listed in Table 30-1, 24 were female and only 17 male. As noted earlier the apparent male predominance in earlier statistics [185] was probably due to the erroneous inclusion of cases of porphyria cutanea tarda which occurs more frequently in males [16].

Of the 41 cases listed in Table 30-1, 16 were observed in families with at least 2 cases [8, 199, 200, 208, 216, 218, 224-226] and in 2 instances 3 cases were reported in the same family, all occurring in a single generation [208, 210-217]. In addition in 5 families, 6 siblings of patients with erythropoietic porphyrin were reported to have died in infancy exhibiting features which may have been porphyrin (these are not included in Table 30-1) [9, 191, 195, 203, 213, 214]. Even without statistical analysis these data clearly show that erythropoietic porphyrin not uncommonly occurs in siblings, a finding which is contrary to a recent statement by Waldenström [16].

In 19 of the cases the family history was reported in sufficient detail to permit estimation of the number of unaffected siblings. The 19 patients with porphyrin were found to have 52 siblings who did not exhibit the disease, a ratio which closely approximates that predicted if the abnormality is inherited as an autosomal recessive gene. In 3 instances, 2 out of 4 siblings were affected [199, 200, 224, 225] whereas in 1 family only 1 out of 12 children had porphyrin [218]. In 3 of the reported families consanguinity of the parents was recorded [194, 200, 219].

The disease has never been observed in successive generations but reproduction is almost nil [16]. The patient reported by Hench et al [197] gave birth to two apparently normal children, one of whom is alive, the other died shortly after a premature delivery. During the first few days of life the living baby excreted considerable amounts of porphyrins which were believed to have been passively transferred through the placenta. This is the only instance in which pregnancy has been reported in a patient with erythropoietic porphyrin. In addition the healthy wife of the famous case Petry had two spontaneous abortions, each in the fourth month of pregnancy [243]. The fetuses were carefully studied but no abnormalities of pigment metabolism were detected [243].

On the basis of the findings it appears probable that porphyrin

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## HEPATIC FORMS OF HUMAN PORPHYRIA

## INTRODUCTION AND CLASSIFICATION

Although in congenital erythropoietic porphyria the metabolic defect can be traced to the developing red cells in the bone marrow where an abnormality in heme biosynthesis accounts for the overproduction of porphyrins of type I isomer such a relationship cannot be demonstrated in the other and more frequently encountered forms of human porphyria. Such patients show no hematologic disorder [18 128] and the porphyrin content of bone marrow and circulating erythrocytes is invariably normal [41 128]. The only organ consistently exhibiting high concentrations of porphyrins or porphyrin precursors or both is the liver [18 28 41 55-59 128 185]. On the basis of these observations it has been proposed to group the latter forms of porphyria together under the heading *hepatic porphyria* as contrasted with the erythropoietic form [18 39 41]. This should not be considered as a final and unalterable classification; it would be much preferable to describe each form of the disease in terms of the metabolic defect involved. Since this stage of knowledge has not yet been reached it appears at least desirable to make a clear distinction between erythropoietic porphyria on one hand and the other less well-defined disorders of porphyrin metabolism on the other hand.

In the hepatic forms of porphyria not only does the liver contain much porphyrin and many porphyrin precursors but impairment of hepatic function or outspoken liver disease is frequent [39 41]. In fact in the photosensitive types of hepatic porphyria liver disease may be so severe as to make it difficult to decide whether the disturbance in porphyrin metabolism is the result of a genetically controlled defect or is a manifestation of advanced hepatic disease [16 38 42 128 137]. This is particularly true of those patients in whom there is no evidence for a familial occurrence of the disorder [38 40 42 43 254 255]. As mentioned earlier there is also reason to believe that a syndrome resembling acute porphyria may develop in supposedly normal persons as a result of addiction to sedatives of the sulfonal and Sedormid group [13 24 26-28 34 36 256]. One may question whether such cases should not better be designated as *porphyrinuria* reserving the term *porphyria* for clearly inherited forms of the disorder. However since in many instances it may be impossible to rule out with certainty a preexisting but unrecognized abnormality of pyrrole metabolism such a distinction would be arbitrary and might better be postponed until more is known of the basic defects involved.

To this problem in deciding whether the metabolic disturbance is inherited or acquired is added the present confusion surrounding the classification of porphyria [16 17 39 137]. Many attempts at a classification acceptable from all standpoints—clinical chemical genetic and

The urine of porphyric cattle contains much uroporphyrin I and smaller amounts of coproporphyrin I [157 212 246 248] in the feces this ratio is reversed [157 212] PBG is usually not demonstrable in the urine nor could it be detected in the liver of an afflicted animal [212, 247 248] It is apparent that bovine congenital porphyria like its human counterpart is characterized by overproduction of porphyrins of type I isomer in cells of the erythroid series The recent finding by Rimington's group [180] that incubation of PBG with hemolyzed erythrocytes obtained from porphyric cattle resulted in formation of both uroporphyrinogen I and uroporphyrinogen III suggests that the metabolic defect in the bovine disease may be similar to that in human porphyria

**Pigs** Congenital porphyria is also observed in pigs [157 246 248 249] but in contrast to the human and bovine forms the porcine disease is probably inherited as a Mendelian dominant trait [157, 246] although further studies on the precise mode of inheritance are necessary Porphyric pigs excrete in the urine large amounts of uroporphyrin and smaller quantities of coproporphyrin and of porphyrins with seven six and five carboxyl groups, the feces contain mostly coproporphyrin [249] Almost all the excreted porphyrins belong to the type I isomer series [157] porphobilinogen is not demonstrable [246] Affected pigs do not exhibit photosensitivity, and the diagnosis rests mainly on the discovery of red fluorescence in bones and teeth Teeth of newborn pigs with porphyria contain considerable amounts of uroporphyrin I [157 246] a finding which serves as a convenient mode of diagnosis during lifetime [246]

Little is known of the biochemical nature and the anatomic site of the metabolic defect It remains to be shown whether the overproduction of porphyrins of type I isomer occurs in erythroid cells as in the human and bovine forms of the disease or whether the pigments are derived from other sites Chemical and microscopic studies of bone marrow have not yet been reported but With [157] observed high concentrations of porphyrins in the spleen and much smaller amounts in liver kidneys and blood Pigs affected with the disease appear to show a considerable degree of fluctuation in the amounts of porphyrins produced [157] In some instances where high concentrations of uroporphyrin were demonstrated in the teeth at birth no evidence for a disturbance in porphyrin metabolism was detectable a few months later at autopsy [157] This has been taken to indicate that the porcine form of porphyria may show a high degree of latency [157] a factor which renders genetic studies very difficult

**Other Species** Finally it should be noted that in at least two species the fox squirrel (*Sciurus niger*) and the touraco bird (*Musophagidae*) uroporphyrin is a physiologic end product of metabolism [250-253] The metabolic origin of the pigment in these species is not known

[16 39] These manifestations are characteristically intermittent in nature. Acute attacks may last from several days to many months. Between acute episodes periods of remission occur during which symptoms may be slight or even absent [18].

Neurologic manifestations are extremely variable. They may involve peripheral nerves, autonomic nervous system, brain stem, cranial nerves, or cerebral function. Extensive reviews of the neurologic and neuropathologic findings have recently been published [16 18 20 187 262 263]. As noted earlier, the relationship of porphyrins and porphyrin precursors to the disturbances of the nervous system is not clear. It is noteworthy that at autopsy the brain and the spinal cord of patients with porphyria usually fail to exhibit PBG or increased concentrations of porphyrins [41 187, 264].

Many of these patients have hypertension, but this is not always present and at times the blood pressure may actually be low [265]. Photosensitivity and increased mechanical fragility of the skin are lacking. The gastrointestinal manifestations frequently lead to weight loss and occasionally to severe emaciation, while prolonged vomiting may cause oliguria and azotemia. Death usually occurs from respiratory paralysis, but uremia and cachexia may be contributing factors.

In many instances the metabolic defect may be present in a latent form so that afflicted persons are entirely free of symptoms or exhibit only vague complaints such as dyspepsia or nervousness [14 16 39]. In such patients careful analysis of the urine frequently reveals excretion of increased amounts of PBG or ALA or both [16 19 39 137]. Latent porphyria may never become clinically manifest or acute attacks may occur at a later date. In patients with latent porphyria acute attacks may be precipitated by the administration of various compounds, notably barbiturates, sulfonamides, and anesthetics [14 20 39]. This is however not an invariable sequence of events, as the author has repeatedly observed patients with latent porphyria who underwent major surgery under general anesthesia without developing symptoms of porphyria.

In most instances acute attacks, unless terminating fatally, are followed by periods of latency during which only a careful search for abnormal urinary metabolites may reveal the presence of the metabolic disturbance. It is not uncommon that during latency the conventional qualitative test for PBG [142] may become negative [16 39] and the diagnosis must be established by quantitative analysis for PBG and ALA [19].

### *Chemical Findings*

The most characteristic finding in this type of porphyria is the urinary excretion of large amounts of PBC [14 266]. As has been discussed, PBG

pathogenetic—have provided little clarification [13 16 23 39 41, 131, 182 257] The reasons for this failure are twofold In the first place one and the same term has often been used to describe syndromes with widely varying symptomatology This is best exemplified by considering the different meanings which *porphyria cutanea tarda* has been given by different investigators The term as originally coined by Waldenström in 1937 [14] was used to describe patients in whom porphyrinuria and photosensitivity developed late in life in the absence of abdominal and neurologic symptoms Today, however, the same author distinguishes three or possibly four different types of *porphyria cutanea tarda* [16] while others [18, 39 41] still use the term in its original restricted sense In addition Rimington [17] states that 'in this condition [*porphyria cutanea tarda*] abdominal and neurological manifestations may be the most prominent features while the skin may be insignificantly affected or indeed appear quite normal' It is obvious that this lack of unanimity in terminology has handicapped the search for a satisfactory classification [40]

A second factor contributing to the present confusion was the discovery that in the same family a genetically controlled disturbance of porphyrin metabolism could give rise to clinical manifestations of widely varying nature, including abdominal neurologic or cutaneous symptoms [187 258] A purely descriptive classification of such a metabolic disturbance is obviously inadequate In the following discussion an attempt will be made to consider the various forms of hepatic porphyria as they appear in genetically homogeneous groups Such an approach meets with a number of limitations the most serious of which is the present ignorance regarding the precise nature of the metabolic defect

#### SWEDISH TYPE OF PORPHYRIA

##### *Clinical Manifestations*

The most extensive study of this type of familial porphyria was undertaken by Waldenström in 1937 [14] although many similar cases had been reported previously [18] The condition has been described as acute porphyria [18 14 16] or as intermittent acute porphyria [18]

Abdominal pain of moderate to severe degree and often colicky in nature is the initial and frequently the most prominent symptom The pain may be generalized or localized but the abdomen is usually soft, and tenderness is not marked Rebound tenderness is lacking A ray examination frequently reveals areas of intestinal distention proximal to areas of spasm [259] Constipation is usually marked and may give rise to confusion with bowel obstruction [260 261] Distention of the stomach and pernicious vomiting are at times outspoken Slight fever leukocytosis, and tachycardia may occur but are not universally present

[16 39] These manifestations are characteristically intermittent in nature. Acute attacks may last from several days to many months. Between acute episodes periods of remission occur during which symptoms may be slight or even absent [18].

Neurologic manifestations are extremely variable. They may involve peripheral nerves, autonomic nervous system, brain stem, cranial nerves, or cerebral function. Extensive reviews of the neurologic and neuropathologic findings have recently been published [16 18 20 187 262 263]. As noted earlier, the relationship of porphyrins and porphyrin precursors to the disturbances of the nervous system is not clear. It is noteworthy that at autopsy the brain and the spinal cord of patients with porphyria usually fail to exhibit PBG or increased concentrations of porphyrins [41 187 264].

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In many instances the metabolic defect may be present in a latent form so that afflicted persons are entirely free of symptoms or exhibit only vague complaints such as dyspepsia or nervousness [14 16 39]. In such patients careful analysis of the urine frequently reveals excretion of increased amounts of PBG or ALA or both [16 19 39 187]. Latent porphyria may never become clinically manifest or acute attacks may occur at a later date. In patients with latent porphyria acute attacks may be precipitated by the administration of various compounds, notably barbiturates, sulfonamides, and anesthetics [14 20 39]. This is however not an invariable sequence of events, as the author has repeatedly observed patients with latent porphyria who underwent major surgery under general anesthesia without developing symptoms of porphyria.

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#### *Chemical Findings*

The most characteristic finding in this type of porphyria is the urinary excretion of large amounts of PBG [14 266]. As has been discussed, PBG



is an obligatory intermediate in the biosynthesis of porphyrins and of heme and small amounts of it are normally excreted in the urine. It is a colorless monopyrrolic chromogen giving an intense red color with Ehrlich's aldehyde (*p*-dimethylaminobenzaldehyde); it has a strong absorption band at 560 m $\mu$  and a weaker one at 525 m $\mu$  [123]. In contrast to the red compound obtained with urobilinogen, the Ehrlich aldehyde compound of PBG is not extractable with chloroform [123]. The concentration of PBG present in normal urine is too small to be detected with the conventional method [143] described by Watson and Schwartz [142]. During acute attacks of porphyria, however, the test is strongly positive and a positive result is usually, but not invariably, obtained during latency. Estimation of PBG excretion during acute manifestations has yielded values ranging from less than 30 to 176 mg per liter urine [187] and from 50 to 170 mg per day [37, 136]. During periods of latency these values may be much lower [19, 37, 137].

In addition to PBG, the urine usually contains large amounts of ALA, another obligatory intermediate in the biosynthesis of heme. During an acute episode, the urine may contain as much as 180 mg ALA per day [37, 136, 137]; during remission the values may be much lower [37, 137].

Although the urinary excretion of large amounts of these two compounds is a typical feature of this form of porphyria, the urine may also contain other porphyrin precursors which give a negative Ehrlich reaction [39, 159-161, 267]. Some of these chromogens are undoubtedly porphyrinogens or their partially oxidized derivatives, porphomethene and porphodimethene (Fig. 30-10). In the urine, all these porphyrin precursors, including PBG and possibly ALA, can be converted to porphyrins [121, 144, 162], but the extent of this process and the nature of the porphyrins obtained depends largely on external factors such as pH, exposure to light and air, and presence of oxidizing substances [121, 122, 128, 144, 268]. Freshly voided urine of patients with acute porphyria may contain little if any increase in uroporphyrin concentration [122]; porphyrins are formed only on standing or during the extraction procedure. This is probably why freshly passed urine frequently is of normal color but darkens on standing in light and air [123]. It should be noted, however, that the color is not all due to porphyrins but in part reflects formation of porphobilin, a brown amorphous oxidation product of PBG [123].

The many extensive studies which have been undertaken in an attempt to define more clearly the nature of the porphyrins present in the urine of patients with acute porphyria or formed on standing [14, 56-58, 122, 162, 268-272] have yielded seemingly controversial results. One can probably reconcile these different findings by realizing that most of the isolated porphyrins are actually formed after the urine has been passed and thus are largely artifactual [121, 122]. There is little doubt that

uroporphyrin of both isomer types is present but the isomer ratio appears to be influenced by the conditions under which precursors are converted to porphyrins. A curious and as yet unexplained finding is that most of the porphyrins are present as metal complex the metal probably being zinc [18-172]. This is in contrast to erythropoietic porphyria where uroporphyrin I is largely excreted in its free form.

Few data are available on porphyrin excretion in the stool. Uroporphyrin has been isolated but the concentration does not appear to be

TABLE 30-3 UROPORPHYRIN CONCENTRATIONS IN LIVER AND URINE BEFORE AND AFTER CONVERSION OF PRECURSORS IN PATIENTS WITH THE SWEDISH TYPE OF PORPHYRIA

| Sex | Age | URO in liver<br>μg/100 g wet weight |       | UPO in urine<br>μg/100 ml |        |
|-----|-----|-------------------------------------|-------|---------------------------|--------|
|     |     | Before                              | After | Before                    | After  |
| F   | 23  | ~3                                  | 133   | 240                       | 1 500  |
| M   | 60  | 23                                  | 804   | 37                        | 2 080  |
| M   | 31  | Trace                               | 445   | 170                       | 7 840  |
| M   | 24  | ~0                                  | 410   | 180                       | 37 000 |
| F   | 23  |                                     | 1 600 | 113                       | 15 800 |
| F   | 67  | 18                                  | 143   | 595                       | 1 475  |
| M   | 40  | 31†                                 | 528   | 305                       | 3 980  |
| F   | 27  | 47†                                 | 131   | PBG present               |        |
| F†  | 17  | 1 090†                              | 3 390 | PBG present               |        |

Liver biopsies and urine adjusted to pH 4 and heated for 15 min in boiling water bath.

† Homogenate tested for PBG and found to be strongly positive.

‡ Specimens stored in deep freeze for 18 months prior to analysis.

Note: URO = uroporphyrin.

SOURCE: R. Schmid et al. [1].

very high [39-41, 58-278]. Fecal excretion of coproporphyrin and protoporphyrin is little if at all increased [15~].

With the exception of the liver tissues obtained at autopsy, usually fail to reveal increased porphyrin concentrations [14, 18, 23, 41, 55, 57, 58, 128]. In some cases uroporphyrin or coproporphyrin or both have been identified and isolated from hepatic tissue [41, 47, 68] but this is not possible in all instances [54]. More important is the demonstration that the liver regularly contains large amounts of PBG [41, 56, 57] while in other organs including the spleen, muscle and in bone marrow this precursor has not been detected [41, 56]. Biopsy studies performed on a larger group of patients with acute or latent porphyria showed that fresh liver tissue contained only insignificant amounts of porphyrins but that a marked increase in uroporphyrin concentration could be obtained after converting precursors to porphyrins (Table 30-3) [41].

These findings indicate that in the Swedish type of porphyria the principal metabolic abnormality is the presence and excretion not of porphyrins but of *porphyrin precursors*. The large quantities of ALA, PBG, and other porphyrin precursors excreted in the urine are probably derived from the liver since this is the only organ in which they can be demonstrated. It appears unlikely that these compounds could have reached the liver from other sites since injection of ALA and PBG does not lead to accumulation of these compounds in the liver of experimental animals [146].

### *Possible Nature of the Metabolic Defect*

If the major biochemical defect in this form of porphyria is assumed to involve overproduction of ALA and PBG, two possible mechanisms have to be considered which could give rise to this metabolic derangement. It is conceivable that the biosynthesis of some heme-containing compound may be blocked and that increased pyrrole formation would result from an attempt by the liver to overcome this block [110]. Alternatively, the defect could be at the ALA level resulting either in increased formation of this compound or in interference with one or more of its metabolic pathways of disposition.

Little formation is available to support the first of these two hypotheses. As noted earlier, the liver contains a number of heme proteins, some of which appear to have a relatively rapid turnover. The metabolism of these compounds has not been investigated in porphyria. In experimental porphyria produced in animals by Sedormid, inhibition of catalase synthesis in the liver has been demonstrated [35], but it is not known how this defect is related to the overproduction of porphyrins and of porphyrin precursors [36]. In human porphyria, the few determinations of liver catalase activity have failed to reveal a significant decrease in enzymatic activity [56, 177]. A further argument against the concept of a block in heme protein synthesis is the absence of significantly increased concentrations of coproporphyrin and protoporphyrin [41]. Increased formation of these compounds would be expected if one of the final steps of heme biosynthesis were blocked (Fig. 30-8).

The second hypothesis, which assumes a defect of formation or metabolism of ALA, is consistent with preliminary findings obtained in studying the pool size and disposition of ALA and PBG [82, 274]. The percentage of labeled ALA converted to urinary PBG was found to be several times greater in porphyria than in normal subjects (Fig. 30-16) [82]. This may indicate an adaptive increase in activity of ALA dehydrase in the liver, but direct evidence for this has not yet been obtained. Moreover, the apparent dilution of administered ALA and of PBG suggested that in porphyria the pools of these compounds are increased (Table 30-4). If this is not simply because of altered permeability of the cells for ALA

it may indicate either an increase in formation of ALA or a decrease in one or more of the nonporphyrin pathways by which this compound can be metabolized (Fig 30-5) [82]

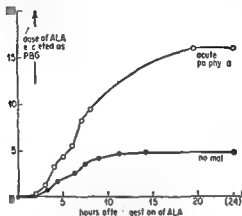


Fig 30-16 Conversion of  $\delta$ -aminolevulinic acid (ALA) to porphobilinogen (PBG) by a normal man and by a patient with acute porphyria. Both were given a dose of 1 gm ALA per 65 kg body weight. Excretion of PBG = expressed as percentage of ALA administered. (By permission of J J Scott [81])

Direct support for this hypothesis is difficult to obtain. It has been observed that in experimental Sideroid porphyria increased formation of porphyrins is associated with decreased purine synthesis [87], a finding which may suggest that an additional amount of ALA is channeled towards PBG production. Similar studies in human porphyria have not yet been reported.

TABLE 30-4 SPECIFIC DILUTION OF ADMINISTERED LABELED ALA BY ENDOGENOUS ALA AND PBG COMBINED

| Subject                                 | ALA<br>type of label | Specific dilution<br>= ratio of ALA and<br>PBG as $\mu$ mole<br>PBG/kg/day |
|---|----------------------|--|
| Normal                                  | [14C]                | 43   |
| Latent I acute porphyria (in remis. on) | [14C]                | 237  |
| Patient II acute porphyria (in relapse) | [14C]                | 334  |

Six millimoles of ALA was administered and the labeling of the excreted PBG was determined.

Source: J J Scott [81]

In *R. spheroides* Shemin and his coworkers [87] have recently obtained evidence for a heat labile inhibitor of ALA synthesis. It was observed that extracts of *R. spheroides* cells grown aerobically in the dark are far

less active for ALA synthesis than extracts obtained from cells grown anaerobically in the light. More important still is the finding that formation of ALA is inhibited when extracts of aerobically dark grown cells are added to extracts from anaerobically light grown cells. This suggests that at the step of ALA synthesis, the cell may possess a regulatory mechanism by which porphyrin and heme biosynthesis can be controlled [87]. If it is permissible to extrapolate these findings to those in acute porphyria, one may speculate that patients with this disease may have a defect involving this regulatory mechanism [87]. Such a disturbance could be limited to the liver or it could be more extensive and include for example, the central nervous system. In the latter case a possible explanation would be provided for the protean nature of the neurologic findings and the patchy anatomic lesions [121]. Proof for these speculations must of course await further investigations.

As noted before increased excretion of ALA and PBG may be detected in asymptomatic carriers of the disease. Furthermore acute attacks of porphyria may be followed by periods of complete symptomatic remission. During latency such patients usually continue to excrete abnormal amounts of PBG and ALA [19-137] but at times the concentration of these metabolites in the urine may return to values scarcely above normal [16-19-39]. This explains not only why during periods of latency patients may be asymptomatic but why it may be difficult to uncover their metabolic derangement. The factors responsible for this fluctuating course are poorly understood although it is well known that barbiturates, sulfonamides and related compounds may exert an aggravating effect [14-20-39].

It has also been noted that the age of onset of symptoms is usually at or shortly following puberty (Fig. 30-17) [16]. manifest porphyria is exceedingly rare before puberty [39-276-278]. In children of afflicted kinships even the presence of latent porphyria is often difficult to detect before adolescence [19]. Furthermore the death rate for porphyria is highest in young adults [16]. These observations indicate that the metabolic defect even though it is inherited may not become clinically apparent until puberty and that symptomatic manifestations are most pronounced during the early part of the reproductive period.

A possible relationship between porphyrin metabolism and gonadal activity is apparent but its nature is not known. In female patients, periodic exacerbations of the disease are sometimes correlated with the menstrual cycle. The author has followed a young woman in whom severe abdominal pain and intestinal obstruction regularly recurred during the premenstrual phase and terminated abruptly with the onset of vaginal flow. For the last 4 years as menstruation has been suppressed by androgenic therapy the patient has remained entirely free of symptoms [177]. The effect of pregnancy on porphyria is unpredictable [279]. In

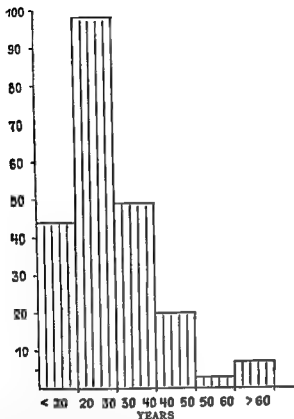


Fig 30-17 Age at first clinical manifestation of Swedish type of porphyria. (By permission of J Waldenström [16])

several instances relapses have occurred during or shortly following delivery [18-39]

### *Heredity and Incidence*

Waldenström's extensive study in Sweden [14, 16, 230] has shown that a positive family history could definitely be established for 243 of 321 proved cases of porphyria. One hundred and thirty seven cases belonged to a single family. The three next largest families contained 14, 12, and 9 porphyric members, respectively. On the basis of these findings, it has been assumed that the metabolic defect is inherited as a Mendelian dominant trait [14, 16, 230]. The frequency with which the disturbance remains asymptomatic and the fact that even genetically certain carriers may fail to exhibit an easily detectable increase in PBG excretion render genetic studies very difficult. A representative pedigree from the Swedish study is given in Fig 30-18. The youngest generation comprised largely

# **SWEDISH TYPE OF PORPHYRIA**

Male Female

□ ○ CLINICALLY NORMAL

▨ ■ CLINICALLY NORMAL, URINE EXAMINED AND FOUND NEGATIVE FOR PBG AND URO

■ ● CLINICAL AND/OR CHEMICAL EVIDENCE OF PORPHYRIA

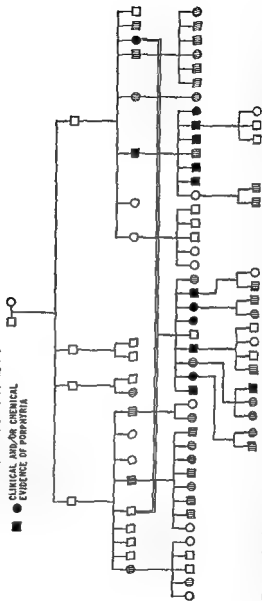


Fig 30-18 Pedigree of a family with the Swedish type of porphyria. Urine of clinically normal individuals was examined with the semiquantitative method for porphobilinogen. It is probable that with the advent of refined methods for estimation of urinary porphobilinogen and  $\delta$ -aminolevulinic acid, more cases of latent porphyria will be discovered. This applies particularly to the fifth generation, which is comprised largely of children [13] (By permission of J. W. Odell and Sm. [16]).

of children : cannot be adequately evaluated because quantitative determinations of PBG and ALA excretion have not yet been reported [19] The occurrence of the disease in several successive generations is a strong indication for its dominant mode of inheritance [16]

Familial occurrence of acute porphyria has been reported by other investigators Watson [39] studied 97 cases and Markovitz [263] collected from the literature 69 cases of acute porphyria many of which had a positive family history Acute attacks of porphyria were observed in two pairs of identical twins [16 231] In earlier studies marked predilection for the female sex was reported [14] Later reports however based on a larger amount of case material yielded a female to male ratio of 3.2 [16 39 263] Latent porphyria on the other hand appeared to be more frequent in males [39] This is consistent with the concept that the metabolic disturbance is inherited as a Mendelian dominant trait but more frequently produces symptoms in young adults of female sex

The incidence of the disease obviously is influenced by its occurrence in large families For Lapland for example Waldenstrom [16] has calculated an incidence of 1/1 000 largely because a single family with no less than 137 known porphyric persons lives in this northern province On the other hand for Sweden proper the incidence is about 1/5 100 000 Judging from the number of reported cases in the literature it would appear that the disease is more frequent in individuals with Scandinavian or English ancestry while in Negroes its occurrence is extremely rare [282 283]

### *Recapitulation*

The Swedish type of hepatic porphyria is characterized by abdominal and neurologic symptoms which are frequently intermittent in nature Cutaneous manifestations are lacking The abnormality is believed to be inherited as a Mendelian dominant trait but afflicted individuals may be free of symptoms Qualitative methods of investigation may fail to reveal the metabolic derangement in asymptomatic persons who are genetically certain carriers

The defect almost certainly involves overproduction of the porphyrin precursors ALA and PBG Increased excretion of porphyrins is variable and may be absent

It has been pointed out [16 137] that acute porphyria and intermittent acute porphyria are synonyms for this condition for the disease is based on a genetically controlled metabolic disorder of chronic nature Furthermore the primary disturbance probably does not involve porphyrin metabolism but occurs at the level of pyrrole biosynthesis which is interrelated with the tricarboxylic acid cycle In future revisions of nomenclature the e factors must be taken into consideration



## SOUTH AFRICAN TYPE OF PORPHYRIA

*Clinical Manifestations*

Dean [284] and Dean and Barnes [137, 258] have studied a large group of porphyric patients in the white population of South Africa. In 13 families a total of 236 porphyric persons were discovered, all of whom could be traced back to a pair of early settlers who immigrated from Holland and married at the Cape of Good Hope in 1688 [258]. Many of these patients exhibit cutaneous manifestations, most pronounced on the hands and face. The skin is sensitive to both sunlight and mechanical trauma, and abrades easily with occasional blister formation. In some of the afflicted individuals skin sensitivity may be slight and intermittent. Cutaneous manifestations are often the only symptoms in men who feel otherwise well throughout life. In some members of this population acute attacks with abdominal pain and paralysis may occur; they are frequently precipitated by barbiturates or anesthetics.

In women skin sensitivity is usually slight, but it may be more pronounced during pregnancy. They are likely to develop recurrent attacks of abdominal pain which may simulate an acute surgical emergency. These patients are usually emotionally unstable, but the diagnosis of porphyria is seldom made before an acute attack of porphyria has occurred. This is likely to happen at some time during adult life and is nearly always precipitated by drugs, especially barbiturates. During an acute episode abdominal pain becomes more severe, peripheral and bulbar paresis may appear, and respiratory paralysis may result in coma and death. Associated with these manifestations is evidence of marked impairment of liver function and excretion of large amounts of PBG and porphyrins in the urine. Acute attacks rarely occur before the age of 16 and are seldom observed after 50; photosensitivity is occasionally present at an earlier age [285]. In many of these persons the disease remains latent throughout life, or abdominal and cutaneous symptoms may be so mild as not to come to a physician's attention.

*Chemical Findings*

In this form of porphyria the characteristic finding is the continuous excretion of large amounts of coproporphyrin and protoporphyrin in the feces. In normal persons upper limits of fecal porphyrin concentration are assumed to be 15 [137] to 20  $\mu\text{g}$  [17] for coproporphyrin and 30 [137] to 45  $\mu\text{g}$  [17] for protoporphyrin, all expressed per gram of dry weight. As seen in Table 30-5, in patients with South African porphyria the concentration of these porphyrins in the feces is greatly increased [137, 220]. Fecal excretion of porphyrins is elevated even though the patients may be in clinical remission or may have been asymptomatic all their lives.

[137] Furthermore increased porphyrin concentration in the stool is demonstrable in asymptomatic children of patients with porphyria [220] As seen in Table 30.6 14 out of 35 such children under the age of 18 were found to have elevated values. It is assumed that these children have latent porphyria which may become manifest during adult life [220]

TABLE 30.5 URINARY ALA AND PBG EXCRETION AND FECAL PORPHYRIN EXCRETION IN THE SOUTH AFRICAN TYPE OF PORPHYRIA

| Sex | Age | Clinical status | Urine                |          |          | Stool (dry) |             |
|-----|-----|-----------------|----------------------|----------|----------|-------------|-------------|
|     |     |                 | PBG qualitative [14] | PBG mg/l | ALA mg/l | COPRO µg/gm | PROTO µg/gm |
| M   | 28  | Acute attack    | +++                  | 194      | 219      | 830         | 1 400       |
| F   | 4   | Acute attack    | +++                  | 99       | 45       | 5 0         | 574         |
| M   | 29  | Acute attack    | ++                   | 40       | 41       | 805         | 810         |
| F   | 35  | Acute attack    | ++                   | 138      | 81       | 673         | 805         |
| M   | 29  | Acute attack    | ++                   | 31       | 30       | 670         | 785         |
| F   | 43  | Mild attack     | +                    | 25       | 26       | 670         | 1 240       |
| F   | 47  | Recent attack   | +                    | 20       | 8        | 345         | 568         |
| F   | 35  | Recent attack   | Neg                  | 6        | ~        | 673         | 397         |
| M   | 21  | Recent attack   | Trace                | 24       | 81       | 580         | 830         |
| M   | 25  | Recent attack   | Neg                  | 9        | 7        | 270         | 508         |
| M   | 57  | Mild pain       | Neg                  | 3        | 3        | 363         | 266         |
| F   | 39  | Mild pain       | Neg                  | 8        | 5        | 477         | 930         |
| M   | 23  | Mild pain       | Neg                  | 6        | 11       | 630         | 760         |
| F   | 35  | Latent          | Neg                  | 2        | 2        | 1 770       | 1 310       |
| M   | 54  | Latent          | Neg                  | 1        | 5        | 106         | 181         |
| F   |     | Latent          | Neg                  | 0        | 1        | 70          | 165         |
| M   | 29  | Latent          | Neg                  | 2        | 3        | 414         | 490         |
| F   | 33  | Latent          | Neg                  | 1        | 1        | 743         | 2 000       |
| M   | 49  | Latent          | Neg                  | 1        | 2        | 159         | 131         |

Note COPRO PROTO = coproporphyrin protoporphyrin respectively

SOURCE: G. Dean and H. D. Barnes [15]

Occasionally photosensitivity is already present during adolescence [283]. In such instances the finding of increased protoporphyrin excretion in the stool may help to differentiate this disorder from erythropoietic porphyria [220].

During clinical remission or in patients with latent porphyria the excretion of porphyrins in the urine may not be significantly increased [137] although exact estimation and analysis of the urinary pigments has not yet been reported. During acute episodes urinary porphyrin excretion is usually much increased [137, 208]. More important is the finding that in patients with acute or subacute symptoms the urine

regularly contains large amounts of ALA and PBG (Table 30.5) while during remission, the excretion of these compounds may return to normal values [137]. As a rule, the occurrence of abdominal and neurologic mani-

TABLE 30.6 PORPHYRIN CONCENTRATION IN THE FECES OF ASYMPTOMATIC CHILDREN OF PARENTS WITH THE SOUTH AFRICAN TYPE OF PORPHYRIA

| Group                     | No. of children | Stool (dry weight)     |        |                        |        |
|---------------------------|-----------------|------------------------|--------|------------------------|--------|
|                           |                 | COPRO $\mu\text{g/gm}$ |        | PROTO $\mu\text{g/gm}$ |        |
|                           |                 | Mean                   | Range  | Mean                   | Range  |
| Normal                    | 23              | 7.5                    | 0-14   | 23                     | 5-51   |
| Abnormal but asymptomatic | 14              | 52                     | 16-158 | 116                    | 51-212 |
| Control normal Bantu      | 13              | 5                      | 1-11   | 10                     | 3-27   |

Note: COPRO, PROTO = coproporphyrin, protoporphyrin respectively.

Source: H. D. Barnes [220].

festations appears to be associated with urinary excretion of porphyrin precursors while increased fecal excretion of porphyrins is present at all times apparently unrelated to the presence or absence of clinical symptoms.

#### Comparison with Other Forms of Porphyria

The high fecal excretion of coproporphyrin and protoporphyrin by white patients in South Africa clearly differentiates this form of porphyria from that studied by Waldenström in Sweden [14, 16, 137]. In the latter group of patients fecal porphyrin excretion is either within normal limits or only moderately increased [137]. In an earlier study it had been suggested that the absence of cutaneous symptoms in Sweden and their presence in male patients in South Africa may be related to increased exposure to sunlight and to mechanical trauma [258]. In a more recent investigation however Dean et al. [137] found Swedish patients whose symptomatology and excretory pattern resemble those seen in South Africa, in that cutaneous symptoms are prominent and the feces contain large amounts of coproporphyrin and protoporphyrin. In South Africa on the other hand a group of patients of British stock, who did not have South African ancestry were found to have features similar to those in the large Swedish families in that cutaneous manifestations are absent and fecal porphyrin excretion is only slightly increased [137]. Furthermore in Holland these authors studied two porphyric families, of which one resembles the South African cases and the other has many features of the disturbance present in the Swedish families [137]. These observations seem to indicate that the differences in clinical and bio-

chemical features are not due to environmental factors but are the result of different genetic patterns

Although the number of different genes responsible for abnormalities in pyrrole and porphyrin metabolism is not known it is conceivable that some of the cases reported as chronic porphyria [12 13 287] mixed porphyria [39 41 286] or porphyria cutanea tarda [17 38] may be related to the type of porphyria present in the white population of South Africa. In two patients with mixed porphyria exhibiting cutaneous abdominal and neurologic manifestations liver tissue obtained by biopsy revealed high concentrations of uroporphyrin [41]. At a later date when photosensitivity was no longer present but abdominal pain was marked a repeat biopsy of the liver in one of these patients revealed little preformed porphyrin but large amounts of porphyrin precursors [41]. Moreover these patients excreted large quantities of PBG and uroporphyrin in the urine at all times. The data presently available do not permit a decision as to whether the disease of these patients is etiologically related to that in South Africa or whether it represents a separate genetic defect.

Similar difficulties are encountered in comparing the South African disease with that which Holtz and Tie [17 288] have described under the term of porphyria cutanea tarda. In their patients increased fecal excretion of coproporphyrin and protoporphyrin is a constant feature but acute attacks with outspoken neurologic manifestations are infrequent and only during such episodes are small amounts of PBG occasionally detectable in the urine. In the South African families on the other hand acute attacks involving the nervous system and at times terminating fatally occur frequently and are invariably associated with excretion of large amounts of PBG and ALA in the urine.

### *Inheritance*

The studies by Dean and Barnes [137 258] indicate that in the white population of South Africa porphyria is inherited as a Mendelian dominant trait. The 236 cases discovered occurred in 13 families all of which had a common ancestry. The disease could be traced through six successive generations.

A representative family tree is given in Fig. 30-19 [258]. The progenitor of this family was born in 1814 and had 478 descendants many of whom were still alive at the time of this study and could be traced. As seen in Table 30-7 there were 60 porphyric patients among the 125 descendants with a porphyric parent excluding children under 18 years of age in the sixth generation. In all cases only one parent had porphyria. In the second generation 5 of the 10 members had porphyria; in the third generation 16 out of 37; in the fourth generation 32 out of 59; in the fifth generation 7 out of the 19 descendants who were over 18 years of age had porphyria.

regularly contains large amounts of ALA and PBG (Table 30-5), while during remission, the excretion of these compounds may return to normal values [137]. As a rule, the occurrence of abdominal and neurologic mani-

TABLE 30-6 PORPHYRIN CONCENTRATION IN THE FECES OF ASYMPTOMATIC CHILDREN OF PARENTS WITH THE SOUTH AFRICAN TYPE OF PORPHYRIA

| Group                     | No. of children | Stool (dry weight)      |        |                        |        |
|---------------------------|-----------------|-------------------------|--------|------------------------|--------|
|                           |                 | COPRO $\mu\text{g/gst}$ |        | PROTO $\mu\text{g/gm}$ |        |
|                           |                 | Mean                    | Range  | Mean                   | Range  |
| Normal                    | 23              | 7.5                     | 0-14   | 23                     | 5-51   |
| Abnormal but asymptomatic | 14              | 52                      | 16-158 | 116                    | 51-212 |
| Control normal Bantu      | 13              | 5                       | 1-11   | 10                     | 3-27   |

Note: COPRO, PROTO = coproporphyrin, protoporphyrin respectively.  
 SOURCE: H. D. Barnes [1970].

festations appears to be associated with urinary excretion of porphyrin precursors, while increased fecal excretion of porphyrins is present at all times, apparently unrelated to the presence or absence of clinical symptoms.

#### Comparison with Other Forms of Porphyria

The high fecal excretion of coproporphyrin and protoporphyrin by white patients in South Africa clearly differentiates this form of porphyria from that studied by Waldenström in Sweden [14, 10, 137]. In the latter group of patients, fecal porphyrin excretion is either within normal limits or only moderately increased [137]. In an earlier study it had been suggested that the absence of cutaneous symptoms in Sweden and their presence in male patients in South Africa may be related to increased exposure to sunlight and to mechanical trauma [258]. In a more recent investigation, however, Dean et al. [137] found Swedish patients whose symptomatology and excretory pattern resemble those seen in South Africa, in that cutaneous symptoms are prominent and the feces contain large amounts of coproporphyrin and protoporphyrin. In South Africa, on the other hand, a group of patients of British stock who did not have South African ancestry were found to have features similar to those in the large Swedish families, in that cutaneous manifestations are absent and fecal porphyrin excretion is only slightly increased [137]. Furthermore, in Holland, these authors studied two porphyric families of which one resembles the South African cases and the other has many features of the disturbance present in the Swedish families [137]. These observations seem to indicate that the differences in clinical and bio-

In the sixth generation all descendants were under 18 years of age in a later study many of these children were found to have increased fecal porphyrin excretion [220]. Thus 48 per cent of the adults with one porphyric parent inherited the disturbance 24 males and 36 females.

Studies of the other 12 porphyric families revealed a similar pattern of inheritance. In the total group of patients 121 were male and 115 were female. Of the adults with one porphyric parent 53.4 per cent exhibited the disease. This conforms with the requirements for a Mendelian autosomal dominant type of inheritance.

#### OTHER FORMS OF FAMILIAL PORPHYRIA

Holtz and his coworkers [17] recently described a porphyric family in Britain in which 10 out of 19 members studied 4 males and 6 females exhibited abnormally high porphyrin excretion. The disturbance was

TABLE 0-7 NUMBER OF MEMBERS IN EACH GENERATION, TOTAL ADULT CHILDREN OF AFFECTED PARENTS, AND NUMBER AND PERCENTAGE OF AFFECTED CHILDREN IN A FAMILY WITH THE SOUTH AFRICAN TYPE OF PORPHYRIA\*

| Generation | Total members | Adult children of affected parent |    |    | Affected |    |    | Percentage affected |
|------------|---------------|-----------------------------------|----|----|----------|----|----|---------------------|
|            |               | No.                               | M  | F  | No.      | M  | F  |                     |
| I          | 1             | —                                 | —  | —  | —        | —  | —  | —                   |
| II         | 10            | 10                                | —  | 5  | 5        | 3  | 2  | 50                  |
| III        | 67            | 37                                | 16 | 21 | 16       | 9  | 7  | 43                  |
| IV         | 173           | 59                                | 17 | 42 | 37       | 10 | —  | 54.2                |
| V          | 61            | 19                                | 3  | 16 | —        | —  | —  | 36.8                |
| VI         | 37            | —                                 | —  | —  | —        | —  | —  | —                   |
| Total      | 410           | 125                               | 41 | 84 | 60       | 24 | 36 | 48                  |

The fifth generation contains many juveniles and young adults and the sixth generation only children. Since fecal excretion of porphyrins has not yet been reported for the members under 18 years of age, the determinations may raise the percentage of affected persons.

SOURCE: Dean et al. [58].

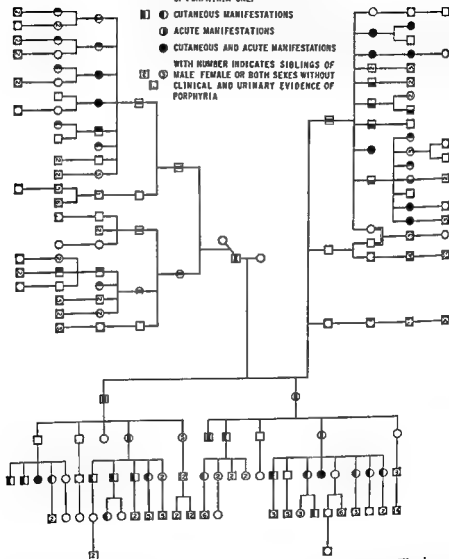
present in four successive generations. Clear evidence for autosomal dominant inheritance was established (Fig. 30-20).

The family member most severely affected was a young woman who had been subject to constipation since early childhood. At various times she experienced attacks of abdominal colic, anorexia, nausea, giddiness, postural hypotension, peripheral neuropathy, and headaches. During pregnancy her symptoms were more pronounced and bullous skin lesions appeared for the first time 1 hr after delivery. Subsequently, areolas of

# **SOUTH AFRICAN TYPE OF PORPHYRIA**

**M to F etc**

- ☐ ☐ CLINICALLY NORMAL
- ☐ ① PATIENTS WITH HISTORY SUGGESTIVE OF PORPHYRIA WHO DIED BEFORE SURVEY
- ☐ ☐ CLINICALLY NORMAL URINARY EVIDENCE OF PORPHYRIA ONLY
- ☐ ① CUTANEOUS MANIFESTATIONS
- ☐ ① ACUTE MANIFESTATIONS
- ☐ CUTANEOUS AND ACUTE MANIFESTATIONS
- WITH NUMBER INDICATES SIBLINGS OF MALE FEMALE OR BOTH SEXES WITHOUT CLINICAL AND URINARY EVIDENCE OF PORPHYRIA



**Fig 30-10** Pedigree of a family with the South African type of porphyria. The diagnosis was established on the basis of clinical manifestations and urinary analysis but fecal examinations for porphyrins have not yet been reported for this family. The last generation is comprised largely of children under 18 years of age many of whom may exhibit increased porphyrin excretion in the feces [0] (By permission of G Dean and H D Barnes [208])

relationship between urinary and biliary excretion of porphyrins [59]. In such patients during remission large amounts of coproporphyrin and protoporphyrin are excreted in the feces. On the other hand during periods of jaundice and liver failure fecal porphyrin excretion is reduced in favor of increased urinary excretion of uroporphyrin and at times of small amounts of PBG. During these latter episodes photosensitivity and abdominal colic are present and neuropathic effects may be observed. It is apparent that in the porphyric family described above [17] such a reciprocity between urinary and biliary excretion of porphyrins was not evident since urinary porphyrin excretion was either within normal limits or was only slightly increased. Furthermore none of the afflicted members exhibited jaundice or liver failure even though cutaneous abdominal and neurologic manifestations were present.

It is difficult to compare this familial form of porphyria with cases of pure porphyria cutanea tarda [18-39-41] with mixed porphyria [39-41-286] or with cutaneous porphyria in the Bantu race [42] in whom the role of genetic factors has not been clearly established. These latter patients often excrete large amounts of uroporphyrin or coproporphyrin or both in the urine and increased urinary porphyrin excretion frequently persists during periods of clinical remission [39-41]. The porphyria in the British family also differs from the hereditary porphyria in the white population of South Africa in that in the latter acute abdominal and neurologic manifestations associated with excretion of large amounts of PBG are much more prominent. Thus the available evidence suggests that the genetically controlled disturbance present in this British family may differ from both the South African and the Swedish types of porphyria and that it may be distinguished from the various cases of chronic or cutaneous porphyria in which a familial occurrence cannot be established.

Similar uncertainty in classification exists in considering other families in whom an apparently inherited abnormality in porphyrin metabolism has been observed. Tio Tiong Hoo [288] studied nine members of a Dutch family (five females four males) all of whom exhibited photodermatitis with blister formation. The first evidence of cutaneous manifestations occurred between 16 and 30 years of age abdominal and neurologic symptoms were consistently absent. Large amounts of coproporphyrin and protoporphyrin were excreted in the feces whereas the urine contained only intermittently small amounts of uroporphyrin and never contained PBG.

Calvy et al [286] reported a family of mixed porphyria in which eight members had bizarre neurologic manifestations in addition to cutaneous lesions. Finally Berger and Goldberg [292] discovered a Swiss family in which four asymptomatic members excreted large quantities of coproporphyrin in urine and stool while uroporphyrin and PBG were not detectable. The abnormality was most pronounced in a 10-year old



skin habitually exposed to sunlight exhibited marked fragility but direct light sensitivity was not detectable. Laboratory studies indicated a slight microcytic hypochromic anemia and mild impairment of hepatic function but jaundice or evidence of liver failure was not present.

Of the other 11 members who exhibited chemical evidence of porphyria only 1 was light sensitive and 11 had noticed an unusual degree of fragility of exposed skin while 3 had no cutaneous manifestations. At one time or other, however, all 9 had experienced mild gastrointestinal and

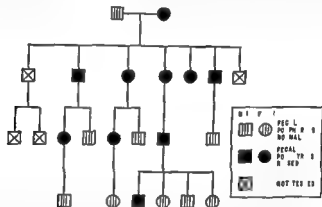


Fig. 30-20 Pedigree showing the dominant autosomal inheritance of porphyria in the family described by Holtz et al [17] (By permission of G. Holtz et al [17]).

neurologic symptoms. Chemically the characteristic finding in this family was a marked increase in fecal excretion of coproporphyrin and protoporphyrin. Elevated values were present both in patients who exhibited porphyric manifestations and in those who were in temporary remission. On the other hand urinary porphyrin excretion was within normal limits or was only slightly increased. The nature and isomer type of the porphyrins in the urine has not been reported, nor are quantitative data available on the excretion of PBG and ALA except that the semiquantitative test for PBG [142] gave at times a slightly positive reaction.

Rimington [17, 128] classifies these patients as having 'porphyria cutanea tarda' and Waldenström [16] and Rimington [289] have proposed the name protocoproporphyria hereditaria. While this latter term may be applicable to the disturbance in this particular family in that the major biochemical abnormality appears to consist in the continuous excretion of large amounts of coproporphyrin and protoporphyrin in the feces, different excretory patterns are observed in other patients with similar clinical manifestations. On the basis of study of a few isolated and apparently nonfamilial cases of porphyria which were described under the term of porphyria cutanea tarda [289, 290, 291] or of chronic porphyria [287] Rimington has postulated the existence of a reciprocal

diagnosed disease (Table 30.8). Its occurrence in more than one child of the same family and the ratio of 1:3 for affected to unaffected siblings of both sexes suggest that the abnormality is inherited as a Mendelian autosomal recessive trait. The penetrance of the abnormal gene appears to be complete since *formes frustes* are not observed. Parents and unaffected siblings of patients fail to exhibit abnormalities in porphyrin excretion.

2. Impressive progress has been made in uncovering the nature of the metabolic defect and the problem appears to be within sight of solution. The major derangement undoubtedly consists of an enzymatic defect in the developing red blood cells which leads to increased formation of porphyrins of type I isomer.

3. For the *other forms of inherited porphyria* (Table 30.8) the situation is less clear. Different genetic types can be recognized but on symptomatic grounds alone a separation is not always possible. Cases of latent porphyria or patients exhibiting only minimal symptoms are frequently encountered. Moreover in many instances the metabolic abnormality cannot be shown to be genetically controlled but may be acquired or may possibly represent a new mutation. Further studies are necessary to clarify the existing confusion.

4. In each patient or genetically homogeneous group of patients with porphyria the excretory pattern of porphyrin precursors and of porphyrins in urine and feces should be studied both during symptoms and during clinical remission. These investigations should be performed with generally accepted methods and should include as many asymptomatic blood relatives as possible. This information would provide an indispensable basis for comparison of the different clinical forms of porphyria. A most promising beginning of such a study has been made by Dean and Barnes [187]. The investigations have permitted a clear separation of the types of porphyria present in the Swedish and in the South African families. More important still is the elucidation of the nature of the metabolic defects involved and it is here that the most promising field for future studies lies.

## ADDENDUM

An additional case of congenital erythropoietic porphyria has recently been published [203]. A female infant presumably of British stock was noticed to pass pink staining meconium and urine during the first days after birth. Hemolytic anemia, splenomegaly, hypertrichosis and erythrodontia were present. Splenectomy performed at the age of 6 months resulted in marked improvement of the hemolytic process and in reduced porphyrin excretion in the urine. Erythropoietic cells in the bone marrow and peripheral blood exhibited the characteristic mor-

boy coproporphyrinuria of a lesser degree was present in both parents and in an aunt.

In all these apparently inherited disorders of pyrrole and porphyrin metabolism the number of different abnormal genes is unknown and little is understood of the nature of the metabolic defects. Moreover it is difficult to determine to what extent clinical and chemical manifestations of the inherited abnormality may be modified by superimposed acquired disturbances such as alcoholism and malnutrition or by environmental factors such as excessive exposure to sunlight. The important role of the liver as a major site of the metabolic derangement is generally recognized [16, 39, 41, 128, 132]. The association of cutaneous photosensitivity with the presence of preformed porphyrins both in the liver and in the excreta [41] is well documented. It has also been noted however that in many patients who excrete large amounts of porphyrins cutaneous manifestations may be absent or the skin may merely exhibit excessive fragility to mechanical trauma but little direct sensitivity to sunlight. The reason for this is not understood. Moreover it is not clear why in some family groups like those reported by Holtz [17] and by Calvy [286] patients may exhibit abdominal colic and neurologic disturbances in the absence of significantly increased PBG excretion. Although there is no evidence to suggest that this compound exerts a toxic effect on the gastrointestinal tract or the nervous system acute attacks are invariably associated with increased excretion of PBG and ALA in both the Swedish and the South African type of hepatic porphyria.

It has recently been suggested [132] that the appearance of preformed porphyrins in the liver and their excretion in bile and urine may reflect an inherited or acquired defect in those cellular enzyme systems which normally prevent porphyrinogens from being oxidized to porphyrins. Such a decrease in cellular reducing potential could result in increased conversion of porphyrinogens to porphyrins. Since the latter cannot be drawn back into the biosynthetic pathway (Figs. 30.8 to 30.10) they escape from the cells and are excreted. While such a mechanism would account for increased formation of preformed porphyrins in the liver it would not explain an overproduction of ALA and PBG. For instance in a patient with mixed porphyria [41] at a time of marked photosensitivity the liver exhibited a high concentration of preformed porphyrins while later when only abdominal and neurologic symptoms were present the liver contained mainly porphyrin precursors. It is possible that in such instances more than one metabolic defect is involved.

## SUMMARY

1 Among the various forms of inherited disorders of porphyrin metabolism *erythropoietic porphyria* stands out as a well defined and easily

phologic and fluorescence-microscopic abnormalities in addition needle like structures believed to consist of precipitated or crystalline porphyrins were present in some of the abnormal normoblasts and non nucleated red cells. The patient has one healthy brother.

Additional familial cases of erythropoietic porphyria have recently been published [294, 295]. According to recent reports from Turkey [296, 297] occurrence of a cutaneous form of porphyria has been observed in some 400 persons who consumed government-distributed wheat contaminated with various fungicides. Preliminary information indicates that the wheat contained hexachlorobenzene and several organic mercury and copper compounds.

A syndrome of cachexia, photosensitivity, bullous skin eruptions, hyperpigmentation and hypertrichosis developed in individuals who had consumed contaminated wheat 1 month to 3 years prior to the onset of manifestations. Urine and feces contained large amounts of uro- and coproporphyrins but increased excretion of urinary porphobilinogen was not observed. Abdominal and neurologic manifestations were absent but there was functional and anatomic evidence of liver disease.

The syndrome appeared to be more frequent in children under 15 years of age but was also found in adults of various racial background who had been exposed to contaminated cereals.

The preliminary reports suggest that this syndrome may represent an acquired form of porphyrinuria and may perhaps be similar to the porphyrinuria seen in the Bantus of South Africa.

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TABLE 30 8 SUMMARY OF THE DIFFERENT TYPES OF HEREDITARY HUMAN PORPHYRIA

| Type                      | Synonyms   | Clinical manifestations  | Clinical findings   | Inheritance                                   |
|---------------------------|--|--|---|---|
| Porphyria erythropoietica | Congenital photosensitive porphyria                        | Photosensitivity hemolytic anemia with pleromegaly   | Large amounts of uroporphyrin I and coproporphyrin I in bone marrow erythrocytes and urine                | Recessive no abortive forms                   |
| Swedish type              | Intermittent acute porphyria porphyria hepatica            | Abdominal colic hypertension central nervous system involvement acute attacks frequent photosensitivity absent | ALA and I BG in liver and urine   | Dominant many cases remain clinically latent  |
| South African type        | Porphyria cutanea tarda mixed porphyria porphyria hepatica | Cutaneous abdominal and neurologic acute attacks frequent in female patients                                   | Copro- and protoporphyrin in liver and feces during attacks ALA I BG and porphyrins in urine              | Dominant many cases remain clinically latent  |
| Other types               | Porphyria cutanea tarda mixed porphyria porphyria hepatica | Cutaneous abdominal and neurologic acute attacks rare  | Increased excretion of fecal porphyrins urinary excretion of porphyrins and porphyrin precursors variable | Probably dominant variable degrees of latency |

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**Part Nine**

**Diseases Manifest Primarily  
in the Blood and the  
Blood forming Tissues**

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## Chapter 31

### Hereditary Spherocytosis

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*James H. Jandl*

In hereditary spherocytosis a hemolytic process is associated with an intrinsic defect in the metabolism and structure of the red cell. The cardinal features of this disease are (1) spherocytosis (2) increased rate of red cell turnover (3) splenomegaly (4) familial occurrence and (5) almost invariably benefit from splenectomy. There are many synonyms for this hemolytic disease the most common of which are congenital hemolytic anemia and congenital hemolytic jaundice.

#### *Incidence*

Although not restricted to any single race hereditary spherocytosis is most frequent in people of European origin. Among those of Northern European descent it is the most prevalent of the hereditary hemolytic disorders. This disease affects the sexes equally and often is manifest in early infancy [1, 2].

#### *Clinical Description*

The major symptoms arise from anemic hypoxia. The severity of the anemia varies from time to time and from patient to patient. As the result of increased red cell destruction a mild degree of acholuric jaundice is usually present although it may be variable or intermittent and tends to be less pronounced in early childhood [2]. Gall tones of the pigment type are common even in childhood [3] and presumably are precipitated from bile containing high concentrations of bilirubin. The spleen is usually palpable but the liver is usually normal in size. Although less frequent than in sickle-cell anemia chronic leg ulcers occasionally develop particularly in adulthood. The ulcers are usually bilateral just above the malleoli and ordinarily disappear after splenectomy. A wide assortment of skeletal abnormalities has been described in patients with hereditary spherocytosis. Some of these are presumably caused by the expanding



morphologically from those appearing in acquired anemias manifesting spherocytosis such as hemolytic anemias caused by 'autoantibodies' or by certain oxidant drugs. In hereditary spherocytosis the red cell mean corpuscular volume is usually in the normal or low normal range while the hemoglobin concentration (MCHC) tends to be above normal. The reverse is usually true of the red cells in acquired hemolytic anemia with spherocytosis. The red cell in hereditary spherocytosis is therefore deficient in surface area [7].

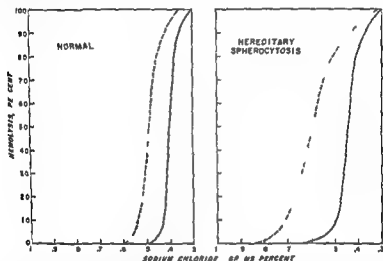


Fig 31-1. Comparison of osmotic fragility curve of normal red cells and of hereditary spherocytes. These are summation curves depicting the portion of red cells hemolyzed at various concentrations of sodium chloride; they represent average values as determined by Emerson and his associates [10]. As indicated by the continuous lines (freely drawn) hereditary spherocytes are on the average slightly more susceptible to hypotonic lysis than normal red cells, and a small portion of these cells is striking in this respect, as indicated by a leftward tail in the osmotic fragility curve. After 24 hr of sterile incubation at 37°C (interrupted line) hereditary spherocytes increase their thickness to diameter ratio more rapidly than do normal red cells, as indicated by the greater leftward shift in the osmotic fragility curve.

Spherocytes are identified by microscopic examination of peripheral blood smears or suspensions in which they appear as dark rounded cells lacking a central pale area and smaller in diameter and greater in thickness than normal red cells. Spheroidicity may be measured in terms of osmotic fragility. In the osmotic fragility test red cells are suspended in aqueous solutions containing various concentrations of sodium chloride. Since there is almost no electrolyte exchange during the relatively short duration of the test, osmotic equilibrium is achieved by the movement of water across the red cell membrane. In hypotonic solution the red cell swells until it approaches a sphere. Any further uptake of water abruptly

force of the hyperplastic erythroid bone marrow during the formative years

### *Pathology*

As in other chronic hemolytic disorders there are compensatory normoblastic hyperplasia of the bone marrow and extension of the red marrow into the mid shafts of the long bones. Occasionally extramedullary erythropoiesis may appear with formation of paravertebral masses. The spleen as observed at operation is darkly congested with blood and usually weighs from 500 to 2 000 gm although a precise weight is often difficult to establish because of a continuing leakage of blood. On section of the spleen the sinuses are often observed to be dilated and empty whereas the pulp cords are congested with red cells. The significance of this finding is uncertain since recent studies [4] of ultrathin sections of the normal spleen indicate that the splenic sinuses and the splenic cords are merely different functional phases of the same anatomic structure.

### *Laboratory Findings*

The presenting laboratory findings are those common to all chronic hemolytic processes: anemia, an increased concentration of reticulocytes, an increased concentration of indirect reacting (largely nonglucuronide) bilirubin in the serum, and an increased rate of fecal urobilinogen excretion. There is little or no hemoglobin in the plasma. The hemoglobin which is released from destroyed red cells is catabolized to bilirubin at the site of destruction (so called extravascular hemolysis).

The severity of the manifestations of the disease depends upon the rate of red cell destruction which may range from slightly to greatly increased and the rate of compensatory red cell production by the marrow. The erythropoietic response to anemia in this disease is unusually brisk. Accordingly the anemia may be minimal despite moderately rapid rates of red cell destruction. Patients having only slight anemia with disproportionately severe icterus and reticulocytosis are said to have a "compensated form" of hemolytic anemia [5]. On the other hand the equilibrium between the rates of red cell destruction and production may be dramatically upset by episodes of bone marrow failure (aplastic crises) during which erythroid hypoplasia and reticulocytopenia may ensue [6]. Most commonly such episodes are precipitated by infections. These infections may be otherwise mild but if highly communicable may cause an epidemic of aplastic crises in an afflicted family.

### *The Spherocyte*

The characteristic feature of this type of hereditary hemolytic anemia is the spherocyte (more accurately a cell more nearly spheroidal than the normal red cell). The red cells in hereditary spherocytosis may differ

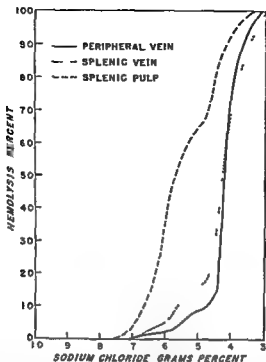


Fig 31-1 Influence of the spleen on the osmotic fragility of hereditary spherocytes. The majority of the red cells were only slightly more spherical than normal. However 10 per cent of the red cells in the peripheral blood (continuous line) represent an apparently separate population which distributes around a maximum increment of osmotic lysis at a sodium chloride concentration of between 0.55 and 0.60 gm per cent. A similar but proportionately larger population of spherical cells was found simultaneously emerging from the splenic vein (dotted line) in the splenic pulp itself (interrupted line). The highly spherical population of red cells constituted a majority of those present. Observations such as this indicate that hereditary spherocytes trapped in the spleen undergo further spherical transformation much as they do on incubation *in vitro* (Fig 31-1).

underlying the spherical shape and the abnormal susceptibility to injury by erythrostatics.

## BIOCHEMICAL ABNORMALITIES OF HEREDITARY SPHEROCYTOSIS

### FUNCTION OF THE NORMAL RED CELL

Although the immature red cell is uniquely concerned with the synthesis of hemoglobin, it is otherwise comparable in its metabolic activity to many other tissue cells. When it matures to an adult red cell shortly after its release into the circulation, its function becomes primarily that of transporting oxyhemoglobin. This function requires the preservation

renders the cell membrane porous causing leakage of intracellular contents including hemoglobin. Accordingly assuming that the cells have a normal internal osmolarity and providing the pH is kept constant the osmotic fragility test is a precise measure of how nearly spherical a cell is at the time of exposure to a hypotonic medium [8]. Under conditions of this test the red cells including the newly formed reticulocytes [9] of patients with hereditary spherocytosis usually manifest an increase in osmotic fragility. Thus spheroidicity is attained at higher concentrations of sodium chloride than is the case with normal red cells (Fig. 31.1).

## MECHANISM OF RED CELL DESTRUCTION

### *Red Cell Survival*

Hereditary spherocytes have a diminished life span in the patient or in the normal subject when the spleen is present but their survival is almost normal in the splenectomized patient or normal subject [10]. Normal red cells survive normally when transfused into patients with hereditary spherocytosis [11]. Thus the red cell defect is intrinsic, is nontransferrable and causes destruction of the cell only in the presence of the spleen.

### *Role of the Spleen*

Although spherocytosis persists after splenectomy the excessive destruction of the spherocyte ceases. The following observations have shown that the spleen is the actual and sole site of abnormal red cell destruction and does not simply exert an intermediate influence: (1) The spleens of patients are characteristically engorged with blood and the bilirubin content of splenic vein blood considerably exceeds that of the peripheral blood [12]. (2) Hereditary spherocytes labeled with  $\text{Cr}^{51}$  and reinjected into patients or into normal subjects are sequestered in the spleen but not elsewhere [13, 14] and prior to actual sequestration their circulation time through the spleen is abnormally slow [15].

Direct observations of splenic blood in hereditary spherocytosis have shown that the red cells in the splenic pulp are more nearly spherical than those in the peripheral circulation [16-19] (Fig. 31.2). Spherocytes are selectively retained in the pulp of spleens as determined after transfusion *in vivo* [16-20] and after splenic perfusion *in vitro* [18]. Indeed it has recently been shown [21] that spherocytes can be separated from normal red cells simply by passage through mechanical (Millipore) filters *in vitro*. These observations support in all essential details the hypothesis of Ham and Castle [22, 23] that hereditary spherocytes are trapped in the spleen by virtue of their increased thickness. There they are subject to hemoconcentration [24] and erythrostasis and undergo metabolic changes to which they are peculiarly susceptible leading to their destruction. In recent years efforts have been made to identify the biochemical lesion

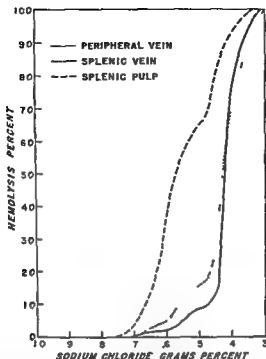


Fig 31.2 Influence of the spleen on the osmotic fragility of hereditary spherocytes. The majority of the red cells were only slightly more spherical than normal. However, 10 per cent of the red cells in the peripheral blood (continuous line) represent an apparently separate population which distributes around a maximum increment of osmotic lysis at a sodium chloride concentration of between 0.55 and 0.60 gm per cent. A similar but proportionately larger population of spherical cells was found simultaneously emerging from the splenic vein (dotted line) in the splenic pulp itself (interrupted line); the highly spherical population of red cells constituted a majority of those present. Observations such as this indicate that hereditary spherocytes trapped in the spleen undergo further spherical transformation, much as they do on incubation *in vitro* (Fig. 31.1).

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### *Role of the Spleen*

Although spherocytosis persists after splenectomy the excessive destruction of the spherocyte ceases. The following observations have shown that the spleen is the actual and sole site of abnormal red cell destruction and does not simply exert an intermediate influence. (1) The spleens of patients are characteristically engorged with blood and the bilirubin content of splenic vein blood considerably exceeds that of the peripheral blood [12]. (2) Hereditary spherocytes labeled with  $\text{Cr}^{51}$  and reinjected into patients or into normal subjects are sequestered in the spleen but not elsewhere [13-14] and prior to actual sequestration their circulation time through the spleen is abnormally slow [15].

Direct observations of splenic blood in hereditary spherocytosis have shown that the red cells in the splenic pulp are more nearly spherical than those in the peripheral circulation [16-19] (Fig. 31.2). Spherocytes are selectively retained in the pulp of spleens as determined after transfusion *in vivo* [16-20] and after splenic perfusion *in vitro* [18]. Indeed it has recently been shown [21] that spherocytes can be separated from normal red cells simply by passage through mechanical (Millipore) filters *in vitro*. The observations support in all essential details the hypothesis of Ham and Castle [22-23] that hereditary spherocytes are trapped in the spleen by virtue of their increased thickness. There they are subject to hemoconcentration [24] and erythrostasis and undergo metabolic changes to which they are peculiarly susceptible leading to their destruction. In recent years efforts have been made to identify the biochemical lesion

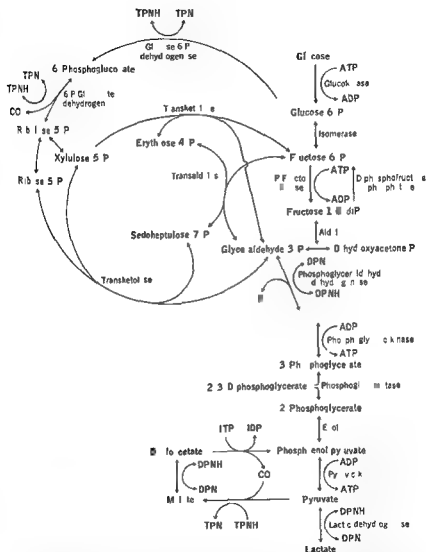


Fig 31.3 Metabolism of glucose by red cells. The main pathway in red cells is the anaerobic (Embden-Meyerhof) pathway portrayed vertically in this scheme. For every mole of glucose consumed in this chain of reactions 4 moles of ATP and 2 moles of inorganic phosphate are utilized; in return 4 moles of ATP are generated and 2 moles of lactate are lost. The cyclc pathway at the upper left represents the hexose monophosphate shunt (pentose phosphate pathway) which serves the important function of generating TPNH.

of (1) hemoglobin molecules and (2) the physical integrity of the cell. The preservation of hemoglobin depends to a large extent upon intracellular reducing mechanisms and involves the formation of reduced triphosphopyridine nucleotide (TPNH). The metabolism of TPNH in the red cell is discussed in Chap. 32. The metabolic activities necessary for the physical preservation of the cell are known to include the generation of high energy phosphate compounds. It is in this respect that hereditary spherocytes appear to be defective.

#### PRESERVATION OF THE PHYSICAL STRUCTURE OF RED CELLS

The survival of the red cell in the circulation is critically dependent upon the preservation of smooth biconcave shape and upon maintenance of individual free suspensibility. In its repetitive passage through the filtering systems of the body of which the spleen is the most exacting, slight alterations in the thickness of the red cell or in the stickiness of its surface lead to sequestration and eventual destruction of the cell.

The supporting structure of the red cell resides in the outer portion of the cell membrane and consists of a membrane [26] or a network [26] of lipoprotein. The protein component, a large complex fibrillar protein termed *elmin* [26], is insoluble and is probably metabolically inactive [27]. The lipid components bound to this insoluble structural protein diffuse from red cells especially under nonphysiologic conditions [28] and exchange rapidly with plasma lipids [29]. Some lipids may be actively synthesized by red cells [30], although this activity is very limited and probably occurs only in the youngest circulating cells [31]. The actual processes which induce and maintain the biconcave structure of the red cell membrane are unknown.

The volume and thickness of red cells depend upon ion transport across the cell membrane. Since distribution of the anions chloride and bicarbonate appears to be passive, maintenance of red cell volume depends largely on the distribution of cations. The movement of cations across this membrane involves energy dependent processes in which potassium is actively concentrated in the cell and sodium is actively discharged. Both ions are in a state of continuous flux [32-35]. The energy for ion transport is derived from glucose metabolism [32-35].

#### METABOLISM OF THE NORMAL RED CELL

##### *Glycolysis*

Energy for the functions of the adult red cell is derived entirely from the metabolism of glucose. Unlike immature red cells including reticulocytes [36], the mature red cell lacks cytochromes and many of the components of the tricarboxylic acid cycle. Since the red cell thus has little ability to utilize oxygen or to metabolize lactic acid, glucose is mainly catabolized

fragility (Fig 31 1) [16 19 22] Swelling is accompanied by a decrease in electrolyte gradients permitting an influx of sodium and a lesser efflux of potassium [32 45] The net gain in electrolytes and water which results may reflect in part that sodium transport is more active than potassium transport [32-35] Indeed after 48 hr of incubation when the intracellular and extracellular concentrations of the cations have both approached equilibrium the red cells return to their original volume [45] However the mechanism of red cell swelling during incubation *in vitro* is still incompletely understood Swelling occurs to about the same extent when the cells are incubated in a potassium rich buffer as in a sodium rich buffer [46] Swelling is presumably due chiefly to oncotic pressure although an increase in intracellular osmolarity [47] may be contributory

During the first several hours red cell osmotic swelling is largely prevented by glucose unless glucose utilization is blocked by the addition of fluoride [32] Red cell swelling caused by prolonged storage is only partially inhibited by glucose and is attended by a depletion of cellular 2,3-diphosphoglycerate [42] and of ATP [48] and the appearance of free purines [49] The changes can be partially reversed by certain ribosides [50] particularly inosine [51] which are cleaved by purine nucleoside phosphorylase [42 52] possibly at the red cell membrane releasing ribose 1 phosphate [53] This ester through its conversion to ribose 5-phosphate presumably could be metabolized to triose phosphate by way of the hexose monophosphate shunt [50] (Fig 31 3) At least some purines may enter the erythrocyte there is recent evidence that adenine itself may also be utilized for ATP synthesis by intact rabbit red cells [54]

## METABOLISM OF HEREDITARY SPHEROCYTES

### *Abnormalities of Ion Transport*

The hemoglobin concentration in hereditary spherocytes frequently exceeds the upper limits of normal Although the exact explanation for this is lacking it is generally attributed to defective electrolyte regulation within the cell Potassium and water content are diminished [55] Although the sodium concentration is normal [46] the rate of sodium turnover is high [56 57]

An even more striking and consistent finding is the crucial observation that these cells are abnormally susceptible to erythrostasis When hereditary spherocytes are incubated at 37 C they become spheroidal even more rapidly than do normal red cells [17] (Fig 31 1) Indeed since the cells are usually partially spherical prior to incubation their exaggerated rate of increase of spheroidicity during *stasis* renders the measurement of osmotic fragility after incubation for 24 hr the most sensitive single test for detecting hereditary spherocytosis [17-19] This exaggerated increase

by the glycolytic (Embden Meyerhof) pathway (Fig. 21.3) and lactate accumulates. In addition to this pathway the mature red cell contains all the enzymatic components of an alternative oxidative pathway, the hexose monophosphate shunt [77] (pentose phosphate pathway of *alv* Chap. 3). This pathway cycles glucose 6-phosphate through several intermediate steps, the first two of which are oxidation in which FPN serves as hydrogen acceptor. Reduced FPN is involved in a number of processes important to the red cell including the reduction of methemoglobin and of oxidized glutathione. When the shunt is active for every three glucose molecules which take the pathway three molecules of carbon dioxide are released and two molecules of fructose 6-phosphate and one molecule of triose phosphate are formed [78]. Ordinarily this pathway is probably little utilized in red cells as indicated by the negligible oxidation of glucose to carbon dioxide *in vitro*. When substances such as methylene blue and other permeable and reversibly reducible dyes which may diffuse into the red cell are added this oxidative shunt is activated to full capacity and cellular respiration is increased dramatically [79, 80]. Such substances substitute for the otherwise deficient mechanism for carrying electron directly from FPNH to oxygen. Possible utilization of the hexose monophosphate shunt *in vivo* has not been quantitated but it is undoubtedly far less active than the anaerobic pathway.

Since the red cell is known to possess only a limited store of glycogen metabolic activity directly depends upon the availability of glucose in the surrounding medium. At physiologic plasma levels glucose can be rapidly transported across the cell membrane so as to maintain equal glucose levels in plasma water and cell water [41]. This transport does not require insulin [41].

### Phosphate Metabolism

Phosphate for participation in red cell metabolism is obtained principally from the orthophosphate of plasma by direct incorporation into glyceraldehyde 3-phosphate probably in the red cell membrane [52, 67]. The phosphorylated triose (1,3-diphosphoglycerate) acts as a phosphate donor in generating adenosine triphosphate (ATP); the more stable diester 2,6-diphosphoglycerate which has a uniquely high concentration in the red cell acts as a phosphate depot. In this respect the diphosphoglycerate occupies a strategic position in red cell energy metabolism comparable to that of creatine phosphate in muscle cells [55].

### Effects of Isotonicity

When normal red cells are incubated *in vitro* under sterile conditions, they undergo progressive structural and chemical changes. During the first 24 hr the cell swell and accordingly show an increase in osmotic

the cell membrane or is merely accentuated by the generally increased rate of intracellular phosphate turnover

### *Effect of Splenectomy*

Although the exact nature of the biochemical defect in hereditary spherocytosis is not known it is apparent that the defect is not merely a consequence of a metabolic change induced by the spleen for the metabolic abnormalities of the spherocytes are not influenced by splenectomy [60]. The failure of splenectomy to affect the abnormal phosphate turnover of hereditary spherocytes does not disprove the possibility that this abnormality is in part acquired in the circulation. The contribution of the spleen to the overall metabolism of the circulating red cells would be difficult to predict and may well be neutralized by the simultaneously opposing factors of (1) increased metabolic stress from splenic erythrostaia (2) the selection of a population of red cells having increased metabolic resistance by virtue of the filtration of spherocytes and (3) the accelerated release of young red cells from the marrow. There remains the possibility that a still more fundamental metabolic error exists in these cells and that phosphate metabolism is only secondarily affected.

## GENETIC ASPECTS

The defect of hereditary spherocytosis is probably inherited as a Mendelian dominant characteristic. This interpretation was first based on surveys which depended upon overt clinical and hematologic manifestations for identification of the defect [64-66] and has been confirmed in studies utilizing more precise laboratory criteria [1, 67]. If the disease is inherited as a Mendelian dominant trait one parent and half the siblings and offspring should be affected. Although abnormal red cells are usually detectable in one parent of each proband a number of instances have been reported in which the red cells of both parents were normal to existing tests [1, 64, 66, 67]. In two large series of patients there has emerged a significant deficit of affected siblings but no deficiency in the expected proportion of affected offspring [1, 66].

There are several possible explanations for the deficit of affected relatives. (1) expression of the disease is highly variable [67] and may be conditioned by such environmental factors as the spleen; there may well be some patients with red cell abnormalities of a mildness undetectable by the osmotic fragility method after incubation of the blood just as there are some with red cell abnormalities undetectable by this method without incubation. (2) some cases may be a result of gene mutation a possibility which would explain the higher incidence in offspring than in siblings and parents. (3) the deficiency in affected siblings may be partly a result of the unusually high rates of miscarriage and infant mortality.

in spheroidicity during erythrostasis is not associated (at least initially) with an abnormal uptake of sodium and water from the surrounding plasma

Since the energy for ion transport depends upon glycolytic mechanisms attention has been focused upon the glucose metabolism of the hereditary spherocyte. The abnormal degree of autohemolysis which ensues when hereditary spherocytes are incubated at 37°C for more than 48 hr can be largely prevented by the addition of glucose [45]. Fluoride nullifies this effect of glucose [48]. As with normal red cells the swelling of hereditary spherocytes exposed to the metabolic stress of erythrostasis is largely prevented by glucose or adenosine and related nucleosides [49].

#### *Abnormalities of Phosphate Metabolism*

The over all rates of glucose consumption [45] and of phosphate exchange [50] in hereditary spherocytes were found to be normal. However the observations of Franker, Altman and Young [50] revealed a consistent abnormality in the intracellular distribution of phosphate. When hereditary spherocytes were incubated in plasma containing  $P^{32}$  labeled orthophosphate there was a disproportionately small recovery of  $P^{32}$  from the intracellular and stromal phosphate esters and a proportionately greater recovery of  $P^{32}$  from the inorganic phosphate pool of the cell. In the red cells of over half the patients studied these workers [50] also found that the addition of adenosine during incubation of hereditary spherocytes with  $P^{32}$  increased toward normal the portion of  $P^{32}$  which could be recovered from phosphate esters. Since Franker et al. interpreted the cellular concentrations of ATP, ADP, 2,3 diphosphoglycerate and inorganic phosphate to be normal they attributed the abnormal distribution of  $P^{32}$  to a diminished rate of turnover of intracellular organic phosphate rather than to changes in the sizes of the respective phosphate pools [50]. Inorganic and ester phosphate levels in the hereditary spherocytosis cell by more recent calculations [51] may be larger than those originally presented [50]. Thus it appears that the movement of phosphate into the various intermediates deviates from normal but the exact nature of this abnormality is presently unclear.

A specific enzymatic defect in hereditary spherocytes has not been described. The finding that the uptake and retention of phosphate by hereditary spherocytes is impaired by a concentration of sodium fluoride ( $10^{-3} M$ ) which has little or no effect upon normal red cells [52] suggests the possibility that  $Mg^{++}$  activated enzymes are involved. One of these enzymes, adenosine triphosphatase, has been found to be normally active in hereditary spherocytes [53] thus abnormalities of both enolase [52] and a phosphokinase [53] have been proposed by exclusion. It is equally possible that the sensitivity of hereditary spherocytes to fluoride poisoning is a function of the structure, permeability, or enzyme placement in

#### 4 One hypothesis which can be proposed is as follows

a An abnormality in intermediary carbohydrate metabolism in hereditary spherocytes renders them peculiarly dependent upon the level of glucose and other metabolites in their environment. Consequently when hereditary spherocytes are separated from a continuous supply of glucose containing plasma as in erythrostasis an abnormally rapid depletion of high energy phosphate compounds occurs ion transport breaks down and the cells increase in spheroidicity at an abnormally rapid rate. This conditioning of the red cells by repetitive passage through the spleen over a period of approximately 10 days [70] creates a minor population of osmotically very fragile thickened red cells.

b With the resulting increase in its thickness the hereditary spherocyte is unable to pass at a normal rate through the splenic sinuses and is thereby subjected to further erythrostasis and erythrocytic concentration which deprive it of metabolic substrate. Being abnormally susceptible to such deprivation the cell becomes even more spheroidal is unable to escape from the spleen and eventually is destroyed by dissolution of the cell membrane.

5 An alternative hypothesis is that structural organization alone may be responsible for the changes found in hereditary spherocytosis and that this abnormality of surface may account for the trapping tendency in the spleen.

6 Hereditary spherocytosis therefore represents an inherited disease of the red cell. The energy requirements of the red cell are relatively so low and its physical deformity of so little consequence in the general circulation that this metabolic defect would be harmless but for the exceptional capacity of the spleen to filter out abnormal cells and to subject them to stasis.

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It has not yet been possible to design experiments which will choose between these alternatives

A number of reports have described the association of hereditary spherocytosis with a variety of constitutional or skeletal defects including tower skull, polydactyly, arched palate, eye abnormalities and ovarian hypofunction [5, 68, 69]. It is not certain, however, that the reported incidence exceeds that of the general population. There is apparently no genetic linkage between hereditary spherocytosis and such inherited constitutional features as sex, blood group, eye color or ear form [60].

There is no definite example of homozygous hereditary spherocytosis although it has been suspected in a family of 13 children, all with hereditary spherocytosis, 9 of whom manifested physical or mental retardation [60]. Race [66] reported a mating of first cousins, both of whom had hereditary spherocytosis. Three of the children were affected, one was normal, and there were two miscarriages.

An interesting observation which emerged from the metabolic studies of hereditary spherocytes was that at least one abnormality observed in hereditary spherocytosis has a familial pattern. It was reported that the defect in intracellular partition of  $P^{32}$  could be largely corrected by adenosine in cells from 10 of 18 patients investigated [60]. In this respect all afflicted members of each family behaved uniformly, regardless of the severity of the anemia [60]. The biochemical basis for this effect of adenosine is unknown, but it cannot be ascribed simply to a deficit in purine nucleoside phosphorylase [60]. This apparent genetic subdivision of patients with hereditary spherocytosis may reflect the existence of two (or more) basic disease entities manifesting in common an inherited spherocytosis.

## SUMMARY

1 Hereditary spherocytosis is a congenital hemolytic anemia caused by an intrinsic defect in the metabolism of the red cell which is transmitted as a Mendelian dominant characteristic.

2 The exact nature of this defect is unknown. It is manifested by the formation in the bone marrow of red cells which are deficient in surface and which thereby approach a spherical rather than a normal discoidal form. This deformity is associated with an abnormality in the metabolism of high energy phosphate compounds by the red cell. Whether this metabolic abnormality is primary or simply secondary to an alteration of the structural organization of the cell membrane is as yet uncertain.

3 Since unlike most body cells, red cells lack intrinsic mechanisms for the direct utilization of oxygen and depend almost entirely on an aerobic glycolysis for the generation of energy, there is rapid depletion of the energy sources necessary for ion transport and for maintenance of cell structure under erythrostatic conditions.

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## Chapter 32

### Drug induced Hemolytic Anemia (Primaquine Sensitivity)

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*Ernest Beutler*

Over the years those charged with the care of the sick have often been dismayed to find that a drug that was innocuous to many patients produced catastrophic results in a few. In 1953 it first became possible to study in detail one such drug sensitivity reaction, the hemolytic effect of the 8-aminoquinoline antimalarial primaquine (Fig. 32-1). These studies not only helped to clarify the mechanism of sensitivity to this drug but also led to the demonstration that a hereditary biochemical lesion of the erythrocyte was responsible for many other drug-induced hemolytic anemias and for favism. Because primaquine sensitivity is the prototype of this kind of hemolytic reaction, only the history of 8-aminoquinoline hemolytic anemia will be reviewed. That of related drug sensitivities is similar.

The history of the 8-aminoquinoline compounds resembles that of many drugs: the compound was hailed as causing no harm to the blood when it was first introduced in 1926; scarcely a year later severe, even fatal, hemolytic anemias were reported. The first 8-aminoquinoline introduced to clinical medicine was plasmoquin (pamaquine) (Fig. 32-1) which was used by Mühlens [1] to treat syphilitic patients who had been inoculated with malaria.

Following Cordes [2] initial report of hemolytic anemia associated with the administration of this drug, many similar reports appeared from all over the world [3-35]. Typically the patient developed dark, often black, urine a few days after primaquine therapy was begun. Jaundice was noted, and the red blood count and hemoglobin were found to be markedly diminished. Usually the patient recovered, but sometimes he succumbed to massive destruction of red blood cells.

Attempts have been made to implicate immune mechanisms in the

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compounds. The clear cut demonstration by Dern et al [38] that sensitivity to the hemolytic anemia induced by these drugs was a property of the red blood cells made it possible to focus attention on the central problem the abnormality of the erythrocyte. These studies demonstrated beyond doubt the important facts that hemolysis is limited to the older members of the red cell population [39] and that red cells sensitive to primaquine are sensitive also to a large number of other compounds [40].

Studies of the biochemistry of primaquine sensitive cells suggested that their sensitivity to hemolysis was related in some way to their glutathione (GSH) content [41] and the stability of their GSH [42]. Examination of the pathways of GSH metabolism in drug sensitive red cells by Carson et al [43] disclosed the probable primary defect a deficiency of the enzyme glucose 6-phosphate dehydrogenase. Subsequent observations established unequivocally that patients with favism have the same red cell defect as primaquine-sensitive subjects [44-56]. Hemolytic anemia has been elicited in a subject with known sensitivity to fava beans by administration of primaquine [57]. In addition it was found that red cells undergoing hemolysis following exposure of individuals to a wide variety of agents had the same biochemical defect as had been described in primaquine-sensitive red cells (Table 32.1).

TABLE 32.1 COMPOUNDS KNOWN TO HAVE INDUCED HEMOLYSIS OF PRIMAQUINE SENSITIVE RED CELLS

|  |  |
|--|--|
| Primaquine                               | Sulfacetamide [ 0 ]                      |
| Paraquine [38]                           | Thiazolsulfone [40]                      |
| Pentaquine [59, 60]                      | Antipyrine [60]                          |
| SN 3883 [59, 60]                         | Irobenecid [61]                          |
| CN 1110 [59, 60]                         | Nitrofurantoin (Furadantin) [62]         |
| SN 15374 [59, 60]                        | Acetylsalicylic acid [63]                |
| Sulfanilamide [40]                       | Furazolidone [64]                        |
| Acetanilid [ 0 ]                         | Sulfamethoxypyridazine (Ilynex) [65]     |
| Phenylhydrazine [40]                     | Sallylazosulfapyridine (Azulfadine) [66] |
| Sulfoxone [60]                           | Naphthalene [5, 63, 67]                  |
| Acetophenetidin (phenacetin) [40, 41]    | 1- $\alpha$ -amino aliphatic acid [68]   |
| N <sub>1</sub> -Acetylsulfanilamide [40] | Ipyramidone [56]                         |

## HEMATOLOGY OF DRUG INDUCED HEMOLYTIC REACTIONS

### CLINICAL COURSE

#### *Result of Drug Administration to a Sensitive Individual*

The clinical course of primaquine induced hemolysis has been studied under carefully controlled conditions by Dern et al [69]. When a drug sensitive person is given 30 mg primaquine daily there is little or no

etiology of hemolytic anemia. A nonspecific hemagglutinin was described in the blood of one patient [21], but it was demonstrated [33] subsequently that autoagglutinins could be found in the blood of patients receiving pamaquine who did not develop hemolytic anemia. Skin testing was attempted [27]. Fragility studies [11, 29, 33, 34] and Donath Landsteiner tests [19, 25] were carried out. In addition, *in vitro* studies were made of hemolysis induced by pamaquine and related compounds or by the plasma

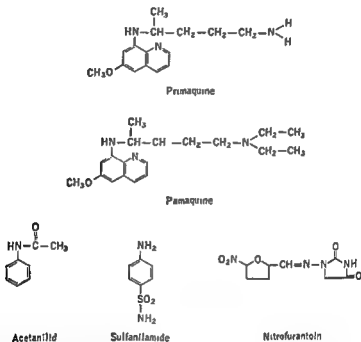


Fig 32 1 Structural formulas of representative types of drugs which cause hemolytic anemia in primaquine sensitive subjects

of patients who had recently received pamaquine [29-30]. None of these studies solved the problem of why some persons develop acute hemolytic anemia when given pamaquine and others do not.

A few observations have been made which in retrospect were much more significant. The appearance of Heinz bodies in the red cells of a patient with mild hemolytic anemia occurring during pamaquine therapy was noted in 1928 [8]. Attention was called to the familial nature of pamaquine sensitivity and analogies were drawn between this disorder and favism [31]. The difference in susceptibility of different races to hemolysis by pamaquine was recognized [18, 22, 25, 27, 29, 33].

It was not until after the introduction of primaquine (Fig 32 1) a therapeutically more effective 8-aminoquinoline [37] that it became possible to study intensively the hemolytic anemia induced by the e

*Hemolytic Anemia in Nonsensitive Individuals*

Volunteer subjects have given an essentially all or none response to daily administration of 30 mg primaquine. On the other hand large doses of those drugs which induce hemolysis in sensitive subjects may induce milder hemolysis in nonsensitive subjects. The hemolytic effect of phenylhydrazine is well known. In addition it has been demonstrated that acetanilid 3.6 gm daily or primaquine 120 mg daily will induce mild hemolysis in a normal person. On the other hand some persons have taken 120 or even 240 mg primaquine daily without any evidence of hemolysis. A combination of 90 mg primaquine and 3.6 gm acetanilid daily produced severe hemolysis in a normal subject [40]. Para amino salicylic acid-induced hemolytic anemia has been described in a subject with nonsensitive red cells as judged by the GSH stability test and assay for glucose 6 phosphate dehydrogenase (see below) [70].

The hemolytic anemia caused by Guadin has an immunologic basis [70]. This drug induced hemolysis may arise by entirely different mechanisms in patients who are not primaquine sensitive.

DRUG SENSITIVITY AS AN INTRINSIC ABNORMALITY OF  
THE ERYTHROCYTE

Dern et al [38] transfused chromium 51 labeled red cells from a sensitive subject into nonsensitive recipients. The survival of the transfused cells was normal until primaquine was administered to the nonsensitive recipient. Rapid destruction of the  $\text{Cr}^{51}$  labeled red cells then occurred. The converse experiment was also carried out. Chromium 51 labeled red cells from a normal individual were transfused into a known primaquine sensitive subject. In contrast to the results obtained in the first experiment primaquine administration had no effect on the survival of the  $\text{Cr}^{51}$  labeled cells from the normal donor although the primaquine-sensitive recipient underwent a typical acute hemolytic episode.

EFFECT OF CELL AGE ON HEMOLYSIS

A curious aspect of primaquine hemolytic anemia is that even if drug administration is continued during a hemolytic episode hemolysis will cease spontaneously. When  $\text{Cr}^{51}$  labeled red cells from a sensitive individual who had become tolerant to primaquine were transfused into a subject who had never previously received primaquine the labeled cells were unaffected when primaquine was given. Red cells from the sensitive subject were no longer susceptible to destruction. When a primaquine-sensitive individual who was no longer destroying his own red cells although still receiving primaquine was given a transfusion of  $\text{Cr}^{51}$



evidence of hemolysis during the first 2 or 3 days. In contrast to immunologic sensitivity, subsequent administration of drug does not shorten this latent period. After 30 mg has been administered daily for 2 or 3 days the urine begins to turn dark. In mild cases the patient may observe no other abnormality. In more severe cases the patient complains of weakness and abdominal and back pain and develops icterus and black urine. Heinz bodies appear in many of the red cells [70]; the hemoglobin, red blood cell and hematocrit values fall rapidly and the number of reticulocytes rises. This 'acute hemolytic phase' ends spontaneously in about 1 week, even when drug administration is continued, and the 'recovery phase' begins. The patient feels better, the color of the urine becomes normal and the hemoglobin, red cell and hematocrit values begin to rise. The reticulocyte count remains high at first and then declines. Throughout this time the Coombs test result is negative and red cell fragility remains unaltered. The only morphologic changes in the blood are polychromasia and the appearance of Heinz bodies in the red cells. Finally, the peripheral blood picture returns to normal and the symptoms vanish, even though administration of the drug is continued in the same dosage that initially caused hemolysis.

Acetanilid and sulfanilamide [69] have also been given under controlled laboratory conditions to subjects known to be drug sensitive. In addition, there are many reports of hemolytic anemia induced accidentally by naphthalene [64, 68-67, 71], nitrofurantoin (Furadantin) [61, 72] and fava beans [47, 73] in persons who were demonstrated to be sensitive or who on clinical grounds may be presumed to have been sensitive. Thus hemolytic anemia induced by acetanilid, sulfanilamide, Furadantin or naphthalene is similar to that induced by primaquine. The self-limited nature of the hemolytic anemia has not been demonstrated, however, with the latter two compounds. Naphthalene-induced hemolytic anemia may at times be considerably more explosive than that observed with primaquine administration.

Fava bean-induced hemolysis may occur within hours of contact with fava beans and is often more severe than primaquine-induced hemolysis. Some factor other than the red cell defect of primaquine sensitivity is required for the production of fava bean-induced hemolysis; in every case of favism in which the red cells have been studied, the erythrocytic defect of primaquine sensitivity has been demonstrated, but subjects known to have the latter defect have eaten fava beans with impunity [47, 74].

Hemolysis induced by thiazol-sulfone may vary greatly in intensity. Careful studies of the mechanism of this variability suggest that it is due to differences in absorption or metabolism of the drug [69]. In addition, Cr<sup>51</sup>-labeled primaquine-sensitive red cells are not necessarily destroyed *in vivo* when fava beans are fed [74a].

## GSH METABOLISM AND ASSOCIATED PATHWAYS IN THE NORMAL ERYTHROCYTE

### OVERALL METABOLISM OF THE ERYTHROCYTE

Several reviews of the metabolism of the mature mammalian erythrocyte have been published in the past decade [78-79]. The metabolism of the erythrocyte which is exceedingly complex may be considered in complete when compared with that of most other cells. For example the mature red cell is unable to synthesize protein but does appear to



Fig. 1. Glucose metabolism in the red blood cell. Abbreviated scheme based on various sources [8-79]. The site of the metabolic block in primary drug-sensitive erythrocytes is indicated by the double line between glucose-6-phosphate and 6-phosphogluconate.

synthesize certain simpler compounds such as glutathione [80-82], nicotinamide mononucleotide and diphosphopyridine nucleotide [82a, 82b]. While it was at one time believed that fat synthesis took place in erythrocytes [83-84], more recent studies indicate that lipid synthesis occurs only in reticulocytes and white cells [84a, 84b]. The red cell requires a source of energy for synthetic processes for the maintenance of ion concentration gradients and for the reduction of methemoglobin which is being formed continuously. The chief source of energy of the red cell

labeled red cells from another primaquine-sensitive subject the transfused cells were destroyed rapidly the sensitive individual retained an undiminished capacity to destroy sensitive cells in his circulation Thus it was demonstrated that the refractory state which develops in primaquine-sensitive individuals is due not to altered metabolism of the drug after prolonged administration, but rather to an alteration in reactivity of the red cell population [69]

It was postulated that the red cell population might consist of a mixture of sensitive and nonsensitive red cells and that sensitivity might be a function of red cell age This hypothesis was confirmed by labeling the red cells of a sensitive subject with  $\text{Fe}^{59}$  By bleeding the subject before administration of the radioisotope and by blocking reutilization with nonlabeled saccharated iron oxide administered intravenously it was possible to confine labeling to red cells of a relatively narrow age range Primaquine was administered when the tagged red cells were 8 to 21 days of age and again when the tagged cells were 63 to 76 days of age Destruction of labeled cells occurred only during the second course of drug administration even though the patient experienced an acute hemolytic episode during both courses of drug administration [39]

#### STUDIES OF DRUG SENSITIVE ERYTHROCYTES BY ROUTINE HEMATOLOGIC TECHNIQUES

The abnormal response of the primaquine-sensitive subject is not another manifestation of a previously described red cell abnormality Coombs tests have been carried out [38 70] The Ham acid hemolyis test has been performed [70] Osmotic and mechanical fragility has been determined [70] Sickle-cell tests have been performed [58 70 77] and the electrophoretic mobility of the hemoglobin has been determined on paper at pH 8.6 [70] The percentages of alkali resistant hemoglobin [70] and methemoglobin [38 58 70] in the red cells have been measured These tests have failed to disclose any relationship between the defect of primaquine sensitivity and other intrinsic red cell abnormalities

Other studies have sought to determine how primaquine-sensitive red cells differ *in vitro* from normal red cells Lysis of primaquine-sensitive cells by primaquine pamaquine 6-methoxyquinoline quinoline and aniline was compared with lysis of normal red cells by the same compounds [70] The accelerating effects of primaquine on saponin hemolysis and of naphthalene on taurocholate hemolysis were measured [70] The effect of prolonged incubation with primaquine on osmotic mechanical and chemical fragility was observed [70] None of these studies showed any difference between primaquine sensitive and primaquine insensitive erythrocytes

### *Reduction of Methemoglobin*

TPNH is utilized for the reduction of methemoglobin through the methemoglobin reductase system [97] in the presence of methylene blue. The available evidence would suggest that this is probably not a physiologically important system [87]. The concept that impairment of TPNH reduction is not important in maintaining normal methemoglobin levels is supported by the finding that primaquine-sensitive cells in the presence of glucose and without methylene blue reduce methemoglobin at a normal rate [65] and by the fact that primaquine-sensitive individuals do not develop greater degrees of methemoglobinemia than normal subjects [33, 58].

### *Synthetic Processes*

TPNH may play an important role in a variety of reductive synthetic processes [98-99]. While some of these probably do not occur in erythrocytes, others may. It may well be that here lies the most important role of TPNH in maintaining erythrocyte integrity.

## GSII METABOLISM

Reduced glutathione (GSII) is a tripeptide of glutamic acid, cysteine, and glycine. It has one free sulfhydryl ( $-SH$ ) group. Recent reviews of the reactions of GSII may be consulted for more detailed description of its chemistry [100-101].

### *Biosynthesis*

Isotopically labeled glycine is incorporated into the glutathione of mature mammalian erythrocytes [80-81]. The finding that all three amino acids are labeled and that the distribution of radioactivity among them remains constant suggests that complete biosynthesis of GSII takes place rather than mere exchange or partial synthesis within the red cell [82].

### *Oxidation*

GSII undergoes oxidation to the disulfide form (GSSG) under a variety of conditions.



In pure solution GSII is relatively stable. In the presence of traces of heme [102], metallic ion, particularly copper [102], and selenite [103], it rapidly undergoes autooxidation to the disulfide form. GSII may be oxidized by hemoglobin peroxide through the mediation of glutathione peroxidase [93]. It appears likely that it can serve as a hydrogen donor

comes from the breakdown of glucose but in vitro inosine may be utilized as well (Fig 32 2)

### *Anaerobic Metabolism*

Under physiologic conditions glucose metabolism in the red cell is primarily anaerobic and yields lactic acid. The derived energy is stored in the form of high energy phosphate bonds by phosphorylation of adenosine diphosphate (ADP) to form adenosine triphosphate (ATP). During glycolysis 1 mole of diphosphopyridine nucleotide (DPN) is reduced in the oxidation of glyceraldehyde 3 phosphate to 1 3 diphosphoglyceric acid and 1 mole of reduced DPN is oxidized in the reduction of pyruvic to lactic acid.

### *Oxidation of Glucose—the Hexose Monophosphate (HMP) Pathway*

An alternate pathway of glucose metabolism is also available. This is the hexose monophosphate shunt (see Chaps 3, 4 and 31) in which glucose undergoes oxidative catabolism. Under normal conditions the HMP pathway accounts for only a small proportion of the glucose utilized by the red blood cell. Methylene blue and certain other dyes readily activate this route of metabolism by linking it to molecular oxygen and to methemoglobin [86-87]. Glucose is phosphorylated at the 6 position and then undergoes oxidation to 6 phosphogluconic acid through the action of glucose 6 phosphate dehydrogenase. In this step 1 mole of triphosphopyridine nucleotide (TPN) is reduced. Phosphogluconic acid then undergoes oxidation presumably forming 6 phospho 3 keto gluconate. Again 1 mole of TPN is reduced. The normal fate of the ribose 5 phosphate formed is less well understood. Various cleavages and condensations may take place [88-89].

### PATHWAYS OF OXIDATION OF TPNH IN THE ERYTHROCYTE

Several alternate routes of oxidation of reduced TPN (TPNH) are available in the red cell. Presumably these reflect some of the physiologic roles of TPNH and of the TPNH generating system in the red cell.

### *Reduction of Oxidized Glutathione (GSSG)*

TPNH is oxidized in the course of enzymatic reduction of oxidized glutathione [90, 91]



It has been suggested that a normal route of electron transfer from TPNH may involve the  $\text{GSSG} \rightleftharpoons \text{GSH}$  reaction [92]. This might occur through oxidation of GSH by peroxide compounds [93-94]. GSH is readily converted to GSSG in vivo. In red cells GSH disappears relatively rapidly when red cells are exposed to oxygen in the absence of glucose [95, 96].

vent the accumulation of peroxide hemoglobin complexes and thereby reduce choleglobin and methemoglobin formation in intact red cells which have been poisoned with azide [94] Yet it is not established that these are physiologic roles

Red cells poisoned with sulfhydryl reagents such as *p* chloromercuribenzoate hemolyze rapidly in vitro [118 119] Such experiments yield no reliable information regarding the need for GSH in the cell since these agents are nonselective in their inactivation of —SH groups It is also known that as the GSH of oxygenated shed blood decreases spontaneous hemolysis is accelerated [90] Since other changes also undoubtedly take place when blood is oxygenated in the absence of glucose the falling GSH and spontaneous hemolysis cannot be considered as having unequivocally a cause and effect relationship

GSH serves as coenzyme for glyoxalase known to be present in red cells [120] but no function is known for glyoxalase in the erythrocyte

It may be as suggested by Barron [121] for other tissues that GSH helps to maintain —SH groups in the reduced state It is of interest that glucose 6-phosphate dehydrogenase [122] and GSH reductase [118 119] both of which serve to keep GSH in the reduced state are inhibited by sulfhydryl reagents Unlikely though it seems it may be that GSH plays no obligatory role in the economy of the red cells Further work utilizing new approaches will be necessary to elucidate the physiologic role of this compound in the red cell

## BIOCHEMISTRY OF DRUG SENSITIVE ERYTHROCYTES

### ABNORMALITIES FOUND INDEPENDENTLY OF DRUG ADMINISTRATION

#### *Glucose 6 Phosphate Dehydrogenase Activity Deficiency*

Dilute hemolyzates from drug sensitive erythrocytes are unable to promote reduction of GSSG normally when glucose 6-phosphate is the substrate Normal GSSG reduction takes place when TPNH or 6-phosphogluconate is supplied This is the basic observation in defining at the enzymatic level the difference between drug sensitive and non-sensitive erythrocytes [43] It indicates that the red cells of sensitive subjects are deficient in glucose 6-phosphate dehydrogenase activity This has been confirmed by many investigators using both the GSH linked assay procedure [50] and an assay method that measures directly the reduction of TPN by hemolyzates supplied with glucose 6-phosphate as substrate [46 51 54 5, 65] Mixtures of various proportions of normal and sensitive hemolyzates do not reveal any enzyme inhibition [122a] The enzyme deficiency seems to be particularly severe in older red cells [123 123a] By using the GSSG linked assay system it has been shown that incubation with a nondialyzable stromal factor present in both sensitive and non

for the reduction of methemoglobin [93, 94, 104] while undergoing oxidation to GSSG. It is apparently oxidized rapidly when red cells are oxygenated [95, 96]. In some tissues GSH may be oxidized by dehydroascorbic acid [105].

### *Reduction*

GSSG may be reduced to GSH nonenzymatically by a variety of agents, including zinc [106], sodium amalgam [107], cysteine [108] and hydrogen sulfide [109] and may also be readily reduced electrolytically [110]. A glutathione reductase [90] which mediates the reduction of the disulfide form with TPNH as the hydrogen donor, has been found in several rat tissues [90, 111] and a number of plant tissues [90, 112]. DPN is ineffective as coenzyme [90, 113].

Human red cells also contain a substance which catalyzes the reduction of GSSG with TPNH serving as a hydrogen donor [91, 114]. Reduced DPN will also serve as a hydrogen donor [91, 114] in the case of red cell GSH reductase although somewhat less efficiently. The relative efficiency of DPNH and TPNH as hydrogen donors has not been found to change in over one thousand fold purification with respect to protein concentration of the red cell enzymes [115]. The degree of specificity of red cell glutathione reductase has not been studied. It is not identical with methemoglobin reductase. Purified preparations of the latter do not promote TPNH oxidation in the presence of GSSG [115]. GSSG reductase can be inhibited with *p*-chloromercuribenzoate without inhibiting methemoglobin reductase [115]. It is entirely possible that glutathione reductase represents the combined activity of several enzymes, and that some of these may be involved also in methemoglobin reduction. This problem deserves further study.

### *Catabolism*

Aside from the interconversion of GSH and GSSG, other pathways for the metabolism of red cell glutathione apparently exist at least in abnormal circumstances. Thus when red cells are incubated with acetyl phenylhydrazine in the absence of glucose, there is a decrease not only in GSH but also (after an initial increase) in GSSG [116, 117]. Since the analytical technique used in these determinations also measures cystine or cysteine, it seems unlikely that some method of breakdown other than simple hydrolysis of the peptide bonds is involved. It has also been suggested that GSSG is destroyed in ordinary hemolyzates [94].

### *Function in the Economy of the Cell*

The function of GSH in the red cell must be regarded as unknown. As has been pointed out, GSH can serve as a hydrogen donor in the reduction of methemoglobin in hemolyzates [93, 94, 104]. It also can pre-

vent the accumulation of peroxide hemoglobin complexes and thereby reduce choleglobin and methemoglobin formation in intact red cells which have been poisoned with azide [94] Yet it is not established that these are physiologic roles

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sensitive cells results in loss of enzymatic activity from sensitive cells from at least some subjects but not from nonsensitive cells [50 124] A stromal factor, therefore appears to inactivate the enzyme in the drug sensitive cells but not in the nonsensitive cells The pH optimum of crude enzyme from normal and sensitive cells has been reported to differ [122a] However this observation has not been confirmed when more highly purified enzyme has been studied [125] Thus the apparent deficiency of the enzyme may in fact be due to an abnormal enzyme molecule which has lower activity and is especially susceptible to damage [122a 126], but much further work needs to be done to determine whether this is the case

There is considerable variability in the severity of enzyme deficiency observed in various subjects In addition recent studies have shown that there is a marked difference between the erythrocyte glucose 6 phosphate dehydrogenase levels of Negro and Caucasian subjects with this enzymatic deficiency The defect is much more severe in affected Caucasian subjects [126a]

Enzyme assays have also been carried out on the leukocytes platelets and liver tissues of sensitive subjects The leukocyte glucose 6-phosphate dehydrogenase activity of Negro subjects with red cell glucose 6-phosphate dehydrogenase deficiency is only slightly reduced and the statistical significance of the reduction is borderline [126a] In the case of Caucasian subjects however a well marked reduction of glucose 6 phosphate dehydrogenase activity was observed in the leukocytes of affected individuals [126b] Reduced platelet enzyme levels have been found in sensitive Caucasian subjects [126b] A borderline decrease in liver enzyme activity in affected subjects has been reported [126c]

#### *Effects of Glucose 6 Phosphate Dehydrogenase Deficiency*

**GSH Deficiency** Red cells from drug sensitive individuals are deficient in GSH and also in total glutathione (GSH + GSSG) [127 128] The GSH deficiency was demonstrated originally in primaquine sensitive subjects both by GSH assay with a nitroprusside method of relatively low specificity and by the use of the more specific alloxan 300 technique [41] Because almost all the nonprotein sulfhydryl of the red cell is GSH [129] the results obtained by means of these two methods were almost identical both yielding low values in drug sensitive and normal values in nonsensitive individuals The deficiency of red cell GSH in drug sensitive individuals is fairly consistent especially if the determinations on several days are averaged [41] There is some overlap of individual values [42] Low GSH values have also been observed in erythrocytes of subjects with hemolytic anemia due to fava beans or drugs other than primaquine [44 45 53 55] It has been claimed that the GSSG as well as the GSH of sensitive erythrocytes is decreased [123] This finding

has been disputed because of the relatively nonspecific assay method for GSSG which was employed [127]

**GSH Instability** There is rapid destruction of GSH [116] when red cells from a sensitive or non sensitive individual are suspended in a saline-phosphate buffer and incubated with acetylphenylhydrazine. Concurrently there is an increase in the red cell GSSG level. Subsequently the GSSG falls slightly, so that a significant decline in total glutathione takes place during the course of the experiment [117]. If glucose or inosine

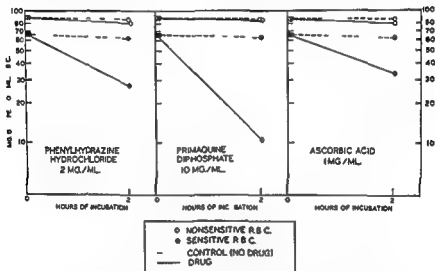


Fig 3-3 The effect of incubation at 37°C with phenylhydrazine, primaquine and ascorbic acid on the GSH levels of sensitive and non-sensitive red cells [130]

is added to the system the non-sensitive red cells protect their GSH completely but protection of GSH in sensitive cells is incomplete [116, 130]. The same effect can be demonstrated also with other reducing agents e.g. primaquine, phenylhydrazine, ascorbic acid (Fig 32-3) [48, 130], Furadantin [61],  $\alpha$  and  $\beta$  naphthoquinone [63] and certain Vitamin K derivatives [63, 67]. Substrates other than glucose and inosine including ribose, lactate, pyruvate, malate and fumarate have little or no protective properties [116]. The addition of methylene blue does not enhance the effectiveness of glucose in protecting red cell GSH [116].

The mechanism by which acetylphenylhydrazine destroys GSH in red cells *in vitro* has been investigated in some detail. Oxygenation of the red cells is required for the destruction of GSH. Carbon dioxide and CO are extremely effective in protecting the GSH of sensitive cells from destruction [116] (Fig 32-4). Acetylphenylhydrazine modifies oxyhemoglobin in such a way that it rapidly oxidizes GSH [116]. The nature

of the intermediate compound has not been determined but it may be that a hemo<sub>2</sub>lobin peroxide compound is formed and that GSH destruction is mediated by GSH peroxidase [94]. It appears, therefore, that acetylphenylhydrazine induces a change in hemoglobin which causes it to oxidize rapidly the GSH of red cells. Normal red cells have the capacity to reduce TPN by oxidizing glucose and to reduce the GSSG to GSH. Normal cells deprived of glucose and cells lacking glucose 6 phosphate

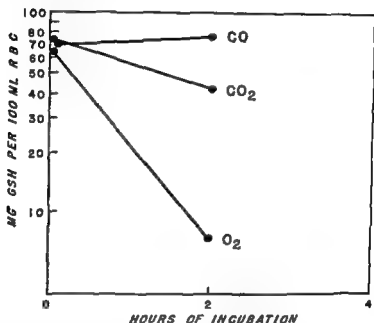


Fig 32-4 The effect of oxygen, carbon dioxide, and carbon monoxide on the GSH content of drug-sensitive red cells incubated with acetylphenylhydrazine. Carbon monoxide and carbon dioxide exerted a pronounced protective effect. (From E. Beutler [48] by permission of the *Journal of Laboratory and Clinical Medicine*.)

dehydrogenase do not reduce enough TPN to maintain GSH in the reduced state. Apparently GSSG is destroyed as it accumulates within the red cell.

An additional complication is that certain hemolytic compounds inhibit purified glucose 6-phosphate dehydrogenase [131]. It remains to be seen what role this may play in bringing about GSH instability in intact red blood cells.

**CO<sub>2</sub> Production and O<sub>2</sub> Consumption** Since the hexose monophosphate shunt is the only known pathway of oxygen consumption and CO<sub>2</sub> production in the red cell [86], an enzyme deficiency along this route would be expected to decrease O<sub>2</sub> consumption and CO<sub>2</sub> production. This is indeed the case when O<sub>2</sub> consumption and CO<sub>2</sub> production of drug-sensitive red cells incubated in the presence of methylene blue are

compared with those of normal cells [123] As expected the amount of  $\text{CO}_2$  produced from the 1 position of labeled glucose was less in drug sensitive than in control cells [123] Furthermore comparison of the utilization of the 1 carbon and the 6-carbon of labeled glucose administered intravenously to drug sensitive and normal subjects has revealed marked retardation of utilization of the 1 carbon by drug sensitive subjects [132] This finding would imply that glucose 6-phosphate dehydrogenase deficiency is not limited to the red cells of sensitive subjects (see below)

**Methemoglobin Reduction** Reduction of methemoglobin in the presence of methylene blue a TPNH linked process is diminished in drug sensitive cells [60]

**TPN/TPNH Ratio** The ratio of TPN to TPNH is increased in sensitive cells [133-135] as might be predicted with a defect in the TPN reducing system A slight increase in the  $\text{DPN}/\text{DPNH}$  ratio has also been described [135]

**Compensatory Mechanisms** A number of other enzymatic changes have also been observed in primaquine sensitive red cells independently of drug administration Increased activity of red cell GSH reductase [92] aldolase [50 134 136] glyceraldehyde phosphate dehydrogenase [55] and lactic dehydrogenase [55] has been reported Activity of the last three enzymes has also been reported to be normal [123 134 136a] Some of these increased activities may represent compensatory changes which enable the red cell to live out a normal life span when not challenged by a hemolytic drug [136]

#### OTHER BIOCHEMICAL MEASUREMENTS IN SENSITIVE RED CELLS

The activity of phosphogluconic dehydrogenase [74] phosphohexose isomerase [74] purine nucleoside phosphorylase [74] triose isomerase [114] 3 phosphoglycerate kinase [134] pyruvate kinase [134] isocitric dehydrogenase [136a] and malic dehydrogenase [136] has also been measured in drug sensitive red cells and has been normal Enolase activity was normal in one subject but decreased in another [134] Chromatography of the amino acid residues of hydrolyzed hemoglobin from sensitive red cells demonstrated no abnormality [48] It has been claimed that a decrease in ADP and AMI as measured by enzymatic hydrolysis occurs in the whole blood of affected subjects [134] Using the more exact chromatographic technique of Bartlett [137] the author and coworker [110] have been unable to confirm this observation (see below) It has also been claimed that intracellular  $\text{K}^+$  of affected cells is significantly diminished [134] No abnormality has been detected in total lipid fractions [134]

Chromatography of phosphorylated intermediary metabolites of the red cells of a drug sensitive individual has been carried out on Dowex 1

TABLE 32.2 RED CELL PHOSPHORYLATED INTERMEDIATES AS SEPARATED CHROMATOGRAPHICALLY ON A DOWEN 1 COLUMN

| Subject  | Glycose<br>6-P <sub>0</sub> | Fructose<br>6-P <sub>0</sub> | Hexose<br>diphosphate | Adenosine<br>monophosphate<br>(AMP) | Adenosine<br>diphosphate<br>(ADP) | Adenosine<br>triphosphate<br>(ATP) | Diphosphoglycerate |
|--|-----------------------------|------------------------------|-----------------------|-------------------------------------|-----------------------------------|------------------------------------|--------------------|
|  |                             |                              |                       |                                     |                                   |                                    |                    |
| Normal 5 subjects (mean $\pm$ 1 s.d.)<br>$\mu$ M/100 ml) | 14.6 $\pm$ 0.6              | 8.7 $\pm$ 4.2                | 44.5 $\pm$ 12.3       | 19.1 $\pm$ 19.3                     | 24.7 $\pm$ 15.9                   | 50.0 $\pm$ 0.5                     | 354 $\pm$ 80       |
| Drug sensitive 1 subject<br>$\mu$ M/100 ml)              | 44.0                        | 10.0                         | 13.5                  | 2.9                                 | 40.4                              | 55.7                               | 391                |

resin [115] employing the techniques developed by Bartlett [137]. The results (Table 32.2) reveal an increase in hexose monophosphate and diphosphate which may readily be explained on the basis of a metabolic block in the direct oxidation of hexose monophosphate. No other abnormalities were observed.

#### CHANGES OCCURRING IN ERYTHROCYTES DURING DRUG ADMINISTRATION

##### *Glucose 6 Phosphate Dehydrogenase*

Only the older members of a red cell population are destroyed when primaquine is administered to a sensitive subject [39]. Studies based on age fractionated red cells indicate that higher levels of glucose 6-phosphate dehydrogenase are present in young cells than in old cells [133-140] and that this disparity exists also when red cells from primaquine sensitive subjects are examined [123, 123a, 126a]. Since a deficiency of glucose 6-phosphate dehydrogenase appears to be the basic defect of primaquine sensitive cells, it would be most attractive to suppose that the higher levels of this enzyme in younger cells protect them against destruction and that the lower levels found in older members of the red cell population render them particularly sensitive to destruction.

Dawson et al. [65] have reported near normal glucose 6-phosphate dehydrogenase levels in subjects with naphthalene-induced hemolytic anemia and Sansone et al. [141] have found normal levels and Grignani and Brunetti [141a] near normal levels in subjects with falcic crises. On the other hand, while measurements of glucose 6-phosphate dehydrogenase activity during the administration of primaquine to sensitive subjects show a slight rise concurrently with the reticulocytosis which follows hemolysis, enzyme activity soon returns to predrug administration levels according to Flanagan et al. [127]. This happens even though the subject is no longer sensitive to the hemolytic action of primaquine. The latter observation is based on the GSH linked assay system and would seem to preclude the attractive explanation that the younger red cells of a drug sensitive individual owe their resistance to hemolysis to higher glucose 6-phosphate dehydrogenase activity and is difficult to reconcile with the findings on *in vitro* age-separated cells [123, 123a, 126a, 133-140]. The discrepancy in these results may be the result of the different assay methods.

##### *GSH*

The administration of primaquine to a drug-sensitive subject results in a rapid fall in the average GSH level of the red cell. This fall precedes the major portion of red cell destruction. There is then a return of average red cell GSH to the same or slightly higher level than prior to drug ex-

hibition [127] Similar observations have been made in subjects with favaism [49 53, 55, 56] and sulfonamide induced [49] hemolytic anemia. It has been suggested that initially GSH in the older red cells is destroyed and that GSH depleted cells are then removed from the circulation [116]

The fate of GSH in sensitive red cells challenged in vivo by drug requires further study. No increase in red cell GSSG has been observed concurrently with GSH destruction in vivo even though many observations have been made at frequent intervals. This has been interpreted as indicating that the route of GSH destruction in vivo does not involve conversion to GSSG [127]. This seems unwarranted, however, since it is entirely possible that the steady state condition of the red cell does not permit GSSG accumulation and that rapid degradation of GSSG takes place as it is formed. It has been reported that the results of the GSH stability test (see below) are not affected by primaquine administration [127]. However transient glutathione instability in red cells of sensitive subjects has been reported following naphthalene [66] and fava bean-induced hemolysis [49 55 141]

### *Methemoglobin Formation*

The formation of methemoglobin in sensitive subjects given drug is not greater than that observed in nonsensitive subjects [33 68] and may even be less [60]

### *Mechanism of Red Cell Destruction*

While much has been learned of the biochemical abnormalities of drug sensitive red cells and of the changes which occur during drug administration comparatively little is known of how these changes lead to red cell destruction when a potentially hemolytic drug is administered.

Hemolytic drugs do not produce preferential lysis of drug sensitive red cells in vitro [70]. Hence they must modify the red cell in such a way that it is selected for destruction presumably by the reticuloendothelial system. While normally the spleen may play a predominant role in the destruction of red cells it has been found that even a splenectomized subject rapidly destroyed drug sensitive red cells transfused into him when drug was administered [38].

Heinz bodies which appear in the red cells at the onset of the hemolytic episode and which disappear as hemolysis progresses may represent a microscopically visible manifestation of the biochemical insult sustained by sensitive cells when drug is administered [70]. The production of Heinz bodies can be reproduced in vitro it is enhanced by sulfhydryl reagents such as arsenite and iodoacetate. Oxygen is required for their formation [142]. Since they are believed to consist of particles of denatured protein [143] it is possible that the insult is one of protein

denaturation occurring under oxidative conditions and that sulfhydryl compounds play a defensive role

Red cell GSH is low in sensitive individuals and falls sharply under conditions of both *in vivo* [127] and *in vitro* [42] exposure to a drug. For this reason this compound has received much attention in attempts at outlining the course of events leading to the destruction of drug sensitive red cells. The suspicion that GSH is involved in this reaction is supported by the disappearance of GSH from cells just prior to their destruction [127] and by the fact that sulfhydryl reagents promote Heinz body formation *in vitro* [142]. Nonetheless sulfhydryl reagents inhibit many enzymatic reactions within red blood cells and it is entirely possible that other more important but less easily measured changes which take place concurrently with GSH destruction may be the primary events leading to red cell destruction. Caution must be taken not to interpret the easily measured GSH changes as being necessarily important in determining destruction of the red cell. As pointed out in an earlier section the role of GSH in the red cell must be regarded as unknown.

### METHODS OF DETECTION OF DRUG SENSITIVE ERYTHROCYTES

The *in vivo* challenge of erythrocytes with a drug is obviously an impractical means of studying the incidence or genetics of drug sensitivity. For such studies it is necessary to depend on *in vitro* means for detection of drug sensitive persons. Several methods for distinguishing apparently normal red cells of drug sensitive subjects from those of normal persons are now available.

#### HEINZ BODIES

When drug sensitive erythrocytes are incubated with a variety of reducing agents such as acetylphenylhydrazine, phenylhydrazine, primaquine or ascorbic acid a different pattern of Heinz body formation is observed than occurs when normal cells are incubated under identical conditions [142]. This forms the basis of a method by which fairly reliable differentiation of sensitive from non sensitive subjects can be made [15, 61, 67, 141, 142]. The chief advantage of this technique is the simplicity with which the test can be performed on large numbers of blood samples. It is also the only method by which positive results are consistently obtained even when the patient is undergoing a hemolytic crisis [127, 141]. Disadvantages include the sensitivity of the test to changes in oxygen tension and to change in the hematocrit [142]. It is not surprising therefore that false positive results have been reported on subjects with thalassemia [141]. Less easily explained are false positive results observed in nonanemic subjects [142].



**Procedure [142]** A buffered acetylphenylhydrazine solution is prepared by dissolving 100 mg acetylphenylhydrazine in 100 ml of a 1/15 phosphate buffer pH 7.6 containing 200 mg glucose per 100 ml. Whole gently agitated venous blood 0.1 ml is added with a 'blow-out' pipet to 2 ml of the acetylphenylhydrazine solution in a test tube with an inner diameter of 12 mm. The suspension is mixed immediately and aerated two or three times by drawing approximately 0.1 ml of the suspension into a pipet and blowing it and a small quantity of air back into the suspension. Incubation is carried out in a 37°C water bath and the mixing procedure is repeated after 2 hr. After 4 hr a small droplet of the suspension is placed on a cover slip which is then inverted on a microscope slide that contains a droplet of half saturated crystal violet in 0.73 per cent NaCl solution. An area in which the structure of the red cells is well preserved is selected for microscopic examination. One hundred cells are examined and are classified as containing either five (or more) or four (or less) Heinz bodies. With rare exceptions over 40 per cent of red cells from sensitive subjects contain five or more Heinz bodies. Less than 30 per cent of red cells from nonanemic non-sensitive subjects contain this many Heinz bodies. Before the blood of anemic patients is to be examined it is useful to centrifuge it slowly and to remove sufficient plasma to adjust the hematocrit to 40 per cent [127].

#### GSH LEVELS

There is a highly significant difference between the average GSH level of sensitive and nonsensitive red cells [41]. Some investigators initially attempted to use this measurement as a means for identifying sensitive subjects [44, 48, 144]. Unfortunately there is too much overlap between the GSH levels of normal and sensitive red cells to make this a useful technique for the detection of drug sensitivity [42].

#### GSH STABILITY TEST

The incubation of red cells with acetylphenylhydrazine and measurement of GSH stability is a useful method for the detection of the red cell defect of drug sensitivity [42, 150]. This test is somewhat more time consuming than the Heinz body method but can be performed relatively simply and rapidly. It is considerably less sensitive to changes in oxygen tension and hematocrit than is the Heinz body procedure. It gives clear separation of sensitive from nonsensitive subjects. A recent report [142] suggests that this procedure is not reliable in detecting females who are sensitive to the *in vivo* hemolytic effects of primaquine but correlation between glucose 6-phosphate dehydrogenase assays and glutathione stability tests has been excellent in the hands of most investigators [51, 54, 55, 141]. Only a few discrepancies between GSH stability tests and enzyme assays have been reported [50, 55, 146]. The author regards this as the method of choice for survey and other routine purposes.

Considerable confusion was caused originally by the observation that red cells obtained from infants up to 75 hr of age had unstable GSH [54, 63, 146-148]. Zinkham [146] has clearly demonstrated that this is

stability is due to rapid depletion of already diminished quantities of blood glucose. Addition of glucose to the system reverses the apparent defect in all but a few infants who prove to have glucose 6 phosphate dehydrogenase deficiency and may therefore be regarded as truly sensitive [146-149].

**Procedure** [42] Venous blood is drawn into heparin or into ACD<sup>1</sup> solution. The use of the latter anticoagulant assures the presence of sufficient glucose

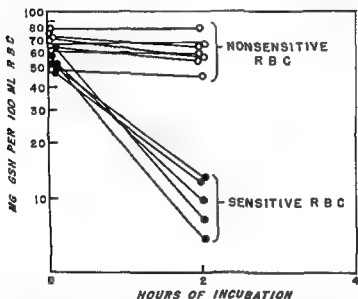


Fig 375 The results of the GSH stability test as carried out on the blood of seven non-sensitive and five sensitive male subjects (E B Utter [1] by permission of the Journal of Laboratory and Clinical Medicine)

■ substrate for GSH reduction. If ACD solution is used acceptable results may be expected even if sterile samples are stored for 4 weeks at 4°C [54-150]. One milliliter of blood is added to a tube containing 5 mg acetyl phenylhydrazine. These are mixed and shaken to ensure thorough oxygenation and incubated for 3 hr at 37°C. At the end of the incubation period 2 ml distilled water is added. Within a few minutes 5 ml of 3 per cent glacial metaphosphoric acid (35 per cent  $\text{H}_2\text{O}_2$ ) is added with agitation followed by approximately 1 gm NaCl and the mixture is shaken. Two milliliters of a filtrate is added to 6 ml of a saturated  $\text{NaCl}$  solution in a 25-mm cuvette with all solutions equilibrated at 20 to 22°C. One milliliter of 2 per cent sodium nitroprusside and 1 ml of a  $\text{NaCN}$  (0.67 M)– $\text{Na}_2\text{CO}_3$  (1.5 M) solution are added to develop color. A spectrophotometer reading is made at 520 mμ within 1 min.

After incubation with acetylphenylhydrazine the red cell GSH of sensitive subjects is usually less than 20 mg per 100 ml. Non-sensitive red cells usually

One liter contains 16 gm sodium citrate, 4.8 gm citric acid, 29.5 gm glucose. One milliliter of ACD is used per 5 ml whole blood.

contain more than 35 mg GSH per 100 ml red cells (Fig 32 5) In most cases the difference in color is sufficient to permit correct interpretation with the naked eye [47] This test is unaffected by a wide variety of disease conditions [60 64]

#### GLUCOSE 6-PHOSPHATE DEHYDROGENASE ASSAY

Demonstration of decreased activity of glucose 6-phosphate dehydrogenase is probably the most reliable and exact means for the detection of drug sensitive red cells It should be recalled that 6-phosphogluconate formed in the assays which have been used will be oxidized further if 6-phosphogluconic dehydrogenase is present This results in the reduction of additional TPN Unless a great excess of phosphogluconic dehydrogenase is present, and this does not appear to be true in red blood cells [150] the amount of TPN reduced will depend upon the level of both phosphogluconic dehydrogenase and glucose 6 phosphate dehydrogenase It must be remembered, therefore that abnormalities in the level of phosphogluconic dehydrogenase can also influence the results in GSSG linked TPN linked and dye-linked assays for glucose 6-phosphate dehydrogenase The latter enzyme is apparently quite stable when red cells are stored in ACD solution No decline in enzymatic activity has been noted in 4 weeks [151] The validity of results obtained on a single sample of red cells which showed a sudden drop in enzymatic activity on the seventh day of storage [140] is to be doubted

#### *GSH linked Assay*

An assay for red cell glucose 6-phosphate dehydrogenase has been described in which the reduction of GSSG to GSH is measured by determining the amount of reduced glutathione in the mixture at various intervals after beginning incubation Glucose 6-phosphate dehydrogenase and glutathione reductase are supplied in the hemolyzate The former is the limiting factor in the reaction

**Procedure [43]** Red cells are washed in saline solution and hemolyzed in 5 vol distilled water for 1 hr at 4 C The hemolyzate is centrifuged at 1400 g for 90 min (maximum temperature 37 C) The cleared hemolyzate is dialyzed for 12 hr at 4 C against 40 vol 0.067 M phosphate buffer pH 7.4 Incubation is carried out at 22 C in a 14-ml reaction mixture buffered to pH 7.4 by 0.036 M tris (hydroxymethylaminomethane) Oxidized glutathione is added to give a final concentration of  $1 \times 10^{-4}$  M TPN  $1 \times 10^{-4}$  M glucose 6-phosphate  $5 \times 10^{-4}$  M and dialyzed hemolyzate 0.5 to 0.0 ml final volume to 14 ml with distilled water The reaction is stopped at the end of 15 min by the addition of metaphosphoric acid and reduced glutathione concentration is determined A modification [92] of this procedure has been described in which the hemolyzate is prepared in the cold The concentration of some of the reactants has been altered and the reaction is carried out at 37 C

### *Spectrophotometric Measurement of the Reduction of TPN*

A more rapid method for the measurement of glucose 6 phosphate dehydrogenase activity in red cells is an adaptation of the procedure described earlier for the determination of this enzyme in other tissues [152]. It is based on the spectrophotometric measurement of the appearance of TPNH in a reaction mixture consisting of glucose 6-phosphate, Tris buffer solution and hemolyzate (Fig. 32.6).

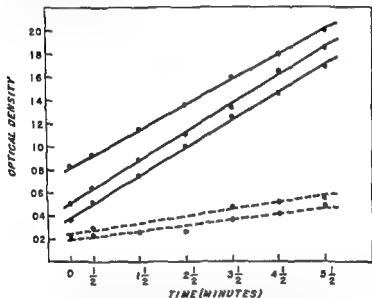


Fig. 32.6. Replicate direct spectrophotometric assays of glucose 6-phosphate dehydrogenase carried out on the red cells of one normal (—) and one sensitive (---) subject. The assay method used was as described by Mark except that 0.5 ml 0.05 M glucose 6-phosphate was added as substrate instead of 0.5 M and that the total volume was increased to 3 ml. Hemoglobin concentration in the two cuvettes was nearly the same. It is apparent that TPN reduction is taking place at a more rapid rate in the preparation from erythrocytes of the normal subject than in the preparation from erythrocytes of the sensitive subject.

**Procedure [150].** After removal of plasma and buffy coat, red cells are washed twice in cold isotonic KCl at pH 7.4 and are lysed by freezing and thawing twice. The assay system consists of 0.1 M  $\text{MgCl}_2$ , 0.5 ml 0.25 M glycylglycine buffer pH 7.6, 0.5 ml  $2.3 \times 10^{-4}$  M TPN, 0.1 ml 0.5 M glucose 6-phosphate, 0.5 ml hemolyzate, water to 2.5 ml. Activity is expressed as  $\Delta \text{O.D.}$  ( $\lambda = 340 \text{ m}\mu$ ) per min per gm Hb or per ml RBC or per 10<sup>12</sup> RBC.

\*The substitution of 0.05 M glucose 6-phosphate is satisfactory and much less expensive.

*Dye linked assay [125 153]*

A quick screening test has been described in which the decolorization (reduction) under oil of brilliant cresyl blue by a hemolyzate supplied with FPN and glucose 6-phosphate is observed with the naked eye. Experience with this test is still limited.

It appears certain that simpler screening methods for the detection of drug sensitive individuals will be developed and that more exact methods for quantitation of enzyme activity such as that of Glock and McLean [122] will be adapted to red cells for more precise studies.

## GENETICS OF DRUG SENSITIVITY

The availability of *in vitro* methods for the detection of the red cell abnormality of drug sensitive subjects has made possible extensive studies of the genetics of this defect. The mode of inheritance has been elucidated beautifully by Childs et al [126].

## DOMINANCE

The defect has been found in as many as three successive generations [74 126] (Fig 32.7 Family 12 LA). For this reason transmission as a recessive characteristic is extremely unlikely.

## SEX DISTRIBUTION

Several curious features have come to light regarding the distribution of the erythrocyte defect between the sexes. It was noted that severe GSH instability or severe glucose 6-phosphate dehydrogenase deficiency occurred most commonly among males [54 65 74 126]. This appeared to support the clinical observation that severe hemolytic anemia on exposure to fava beans occurs more frequently among men than among women [47]. It was also found that intermediate degrees of GSH instability occurred among female relatives of sensitive subjects [54 74 126 154] and that the females usually had intermediate degrees of enzyme deficiency [54, 66 74].

## GENETIC BASIS OF THE SEX DISTRIBUTION

It is apparent from the observations that the genetics of this defect is not simple autosomal inheritance; the defect is either sex linked or sex limited. The available pedigrees reveal that almost invariably it is the mother of affected males who carries the disorder if it is demonstrable in either parent [47 54 74 126]. This finding makes it most probable that the defect is sex linked rather than sex limited [126]. The latter possibility should not result in any alteration in the ratio of mothers and fathers of propositi affected.



TABLE 32.3 INCIDENCE AND DISTRIBUTION OF DRUG SENSITIVITY

| Ra    | S b g p               | Typ f<br>d t | N f<br>a b j t e | I c d e f<br>st st v<br># t g a | R f r           |
|-------|-----------------------|--------------|------------------|---------------------------------|-----------------|
| C i   | U t h t p e           | S- m q       | 1 398            | 0 1                             | [10 158]        |
|       | S d n e p e           | S a m q      | 1 043            | 1 0                             | [17]            |
|       | P u e j b e           | S- a m q     | 00               | 13 5                            | [27]            |
|       | I n d a n t r p e     | S- m q       | 1 915            | 0 3                             | [18]            |
|       | A s h k s e J w e     |              |                  |                                 |                 |
|       | M i                   | GSH St       | 203              | 0                               | [74]            |
|       | F m l e               | GSH St       | 85               | 0                               | [4]             |
|       | N n A s h k J w h i   | GSH St       |                  |                                 | [74 149]        |
|       | I r a q               |              | 89               | 23 6                            |                 |
|       | Y e m                 |              | 6                | 8 1                             |                 |
|       | N t h A f r a         |              | 43               | 2 3                             |                 |
|       | P a                   |              | 9                | 0                               |                 |
|       | E g y p t             |              | 10               | 0                               |                 |
|       | I d                   |              | 1                | 0                               |                 |
|       | T k y                 |              | 2                | 9 1                             |                 |
|       | B l g n               |              | 9                | 0                               |                 |
|       | O t h d k w n         |              | 2                | 0                               |                 |
|       | N A h l e J w i f l e | GSH St       |                  |                                 | [74 149]        |
|       | I q                   |              | 91               | 4 1                             |                 |
|       | Y m n                 |              | 50               | 0 0                             |                 |
|       | N t h A f             |              | 40               |                                 |                 |
|       | F a                   |              | 9                | 41 4                            |                 |
|       | E g y p t             |              | 19               | 0                               |                 |
|       | T k y                 |              | 2                | 4 5                             |                 |
|       | B l g a               |              | 11               | 9 1                             |                 |
|       | O t h a a d k n w n   |              | 1                | 0                               |                 |
|       | A r a b s             | GSH St       | 56               | 3 4                             | [74 149]        |
|       | S d n e               |              |                  |                                 |                 |
|       | M l e                 | GSH St       | 61               | 13 1                            | [100]           |
|       | F m l e               | GSH St       | 38               | 0 (10 5)†                       | [100]           |
|       | I d e                 |              |                  |                                 |                 |
|       | M l e                 | Dye          | 15               | 13 3                            | [103]           |
|       | F m l e               | Dy           | 1                | 100 0                           | [103]           |
|       | I t l e (L g n )      | GSH St       | 100              | 0                               | [100]           |
|       | M p h d               |              |                  |                                 |                 |
|       | M i                   | GSH St       | 30               | 0                               | [48 117]        |
|       | F m l                 | GSH St       | 71               | 0                               | [48 117 122]    |
|       | N t p f i e d         | G 6-pd       | 151              | 1 3                             | [127]           |
|       | N t p h d             | S m q        | 23 9 2           | 0 12                            | [30 33 1 2 102] |
| Negro | S d n e l l e         | S- m q       | 100              | 0                               | [103]           |
|       | B t                   | GSH St       | 100              | 2 0                             | [104]           |
|       | A m N g o e           |              |                  |                                 |                 |
|       | M a l                 | S m q        | 301              | 0                               | [20 22 77 141]  |
|       |                       |              |                  |                                 | [105]           |
|       |                       | GSH St       | 308              | 11                              | [48 11 122 14]  |
|       | F m l                 | S a m q      | 69               | 8 7                             | [145]           |
|       |                       | G H St       | 448              | 2 7 (7 6)†                      | [42 117 122 14] |
|       | H p e e f i d         | G 6-pd       | 152              | 3                               | [127]           |
|       | C h                   | S- a m q     | 38               | 5 3                             | [12]            |
| M e l | M i                   | GSH St       | 41               | 0                               | [106]           |
|       | F m l                 | GSH St       | 36               | 0                               | [106]           |
|       | F m e                 | S m q        | 5                | 12                              | [9]             |
|       | H t l d t             | S a m q      | 9                | 11                              | [11]            |
|       | T h - m l             | GSH St       | 9                | 11                              | [106]           |
|       | J p e e               |              |                  |                                 |                 |
|       | M l e                 | GSH St       | 6                | 0                               | [106]           |
|       | F e m l               | GSH St       | 2                | 0                               | [106]           |
|       | N g n M k t o         | S m q        | 200              | 0                               | [107]           |
|       | P n e                 |              |                  |                                 |                 |
| I d n | M l                   | Dy           | 154              | 0                               | [107]           |
|       | F m l                 | Dy           | 74               | 0                               | [107]           |

† I c d g t m i a t t

Th m thod by wh h n d f n t y w d t e m a d S m q = S- m q l d g  
 adm n t t w h h m l y d p t GSH St = g l t h t a b i l i t y t e t G 6 p d = a s a y f  
 red l i g l o 6- p h p h t d h y d g n Dy = d y h k d s c a t t

available pedigrees show clearly that this is not the case. Thus some females with marked GSH instability and glucose 6 phosphate dehydrogenase deficiency must be heterozygous because they have borne boys who are not affected [cf Fig 32 7 Family K II—A K and E K have produced four negative sons (III—J K P K Go K and G K)]. Further the fact that neither parent of some propositi has the defect demonstrable *in vitro* indicates that the gene can be carried without causing a detectable disorder of the red cell which is demonstrable *in vitro*. Interestingly it has been found that the erythrocytes of at least some such individuals with a negative GSH stability test undergo hemolysis when primaquine is given [14]. The glucose 6 phosphate dehydrogenase content of the red cells of such patients has not been reported.

#### GLUCOSE 6 PHOSPHATE DEHYDROGENASE ACTIVITY IN CELLS OTHER THAN ERYTHROCYTES

Because glucose 6-phosphate dehydrogenase deficiency is genetically determined it would appear reasonable that the synthesis of this enzyme might also be impaired in tissues other than the red blood cell. Studies based on the intravenous administration of specifically labeled glucose show retarded appearance as  $\text{CO}_2$  of the 1 carbon of glucose given to sensitive subjects and thus appear to support this supposition. Only a very limited amount of direct data on enzyme activity in cells other than erythrocytes is available however. Liver biopsies from two sensitive subjects showed less enzyme activity than those of four nonsensitive controls. The difference was much smaller than that observed when red cell enzyme activities were compared. The glucose 6-phosphate dehydrogenase activity of the leukocytes of 14 sensitive subjects did not differ appreciably from that of 31 controls [120c]. Blood platelets from sensitive subjects in contrast have a marked reduction in glucose 6 phosphate dehydrogenase activity [120b]. These findings are compatible with the concept that the enzyme produced by sensitive subjects is abnormal and is more susceptible to damage than normal enzyme [12a]. Nucleated cells such as leukocytes and liver cells could maintain enzyme activity at normal or near normal levels by continued resynthesis but non nucleated cells such as platelets and red cells unable to synthesize more enzyme as they aged would become progressively enzyme-depleted.

#### INCIDENCE AND DISTRIBUTION OF DRUG SENSITIVITY

Two types of information are available on which to base estimates of the incidence and distribution of glucose 6-phosphate dehydrogenase deficiency. The first is the evaluation of reports in the literature of acute hemolytic anemia following the ingestion of drugs. The second consists of



population surveys using the methods developed for *in vitro* detection of the red cell abnormality of sensitive individuals

Some of the more significant information is summarized in Table 32.3. The highest incidence in a single group is in a relatively small series of Iraqi Jews. Twenty per cent of this population has the defect. Other groups of Sephardic Jews have a lower but still quite appreciable incidence. High incidence has been consistently reported in American Negroes among whom the gene frequency seems to be approximately 10 per cent judging from incidence of positive *in vitro* tests and incidence of hemolysis in males. Presumably the incidence would be similarly high in West Africa. A positive GSH stability test result has been demonstrated in a native Nigerian woman presently a student in the United States [117]. The failure to observe hemolysis among Sudanese villagers given pamaquine and the 2 per cent incidence of positive GSH stability test results among Bantu natives lead one to conclude that the incidence of the defect is lower in certain parts of Africa. The incidence of the defect is appreciable in India, Formosa and perhaps Thailand, but lower among the Chinese. Information about the distribution of the defect but not its incidence may be obtained from a recent exhaustive review of the distribution of favism by Sansone et al. [47]. In addition to numerous reports from Sardinia, cases have been reported from almost every region in Italy, from Greece, Israel, Lebanon, Turkey, Spain, Bulgaria and even from West China.

Because the distribution of the defect parallels roughly the distribution of malaria, it has been suggested that glucose 6-phosphate dehydrogenase deficiency like sickle cell hemoglobin may confer resistance to malaria [163]. Preliminary studies appear to support this point of view [167b].

### A CLOSELY RELATED DEFECT

When drug sensitive erythrocytes are not challenged with drug their survival is normal. Observations by Newton et al. [163, 169] confirmed by others [170, 171] suggest that certain subjects who clinically are suffering from nonspherocytic hemolytic anemia have the same enzymatic abnormality as drug sensitive subjects. In common with drug sensitive subjects their red cells have unstable GSH and have markedly diminished glucose 6-phosphate dehydrogenase activity. Heinz body formation in their red cells after incubation with acetylphenylhydrazine is also identical with that seen in drug sensitive subjects. They differ from drug sensitive subjects in that shortening of red cell survival as measured by the  $\text{Cr}^{51}$  technique occurs in these subjects and in their mothers [169, 171]. Furthermore this disorder differs from drug sensitivity in that all the individuals affected have been Caucasian (in one case of Irish stock). Other cases of hemolytic anemia reported recently [133] may fit into

this category. It seems most likely that this form of nonspherocytic hemolytic anemia represents the effect of another much rarer genetic mutation which also affects glucose 6-phosphate dehydrogenase activity, but perhaps through another mechanism and perhaps to a much greater degree.

## SUMMARY

1 When certain ordinarily harmless drugs are administered to susceptible persons, an acute hemolytic reaction results. Among these are 8-aminoquinoline antimalarials such as primaquine, certain sulfonamides, acetanilid, phenacetin, Iuradantin, and many others. Persons with a history of favism have the same red cell abnormality.

2 When sensitive persons receive the drug, the older members of the red cell population are rapidly destroyed. The hemolytic anemia then ceases, even while drug administration is continued, because young erythrocytes are resistant to destruction.

3 Susceptibility to drug-induced hemolysis is due to an intrinsic abnormality of the erythrocyte.

4 Drug-sensitive red cells have reduced glucose 6-phosphate dehydrogenase activity. This enzyme normally catalyzes the oxidation of glucose 6-phosphate, which is coupled to reduction of TPN. TPNH in turn maintains glutathione in its reduced form (GSH). Drug-sensitive red cells are unable to reduce TPN at a normal rate. Probably as a result of this enzymatic defect, the GSH content of sensitive cells is decreased, and when sensitive cells are challenged with drug *in vivo* or *in vitro*, a further decrease in GSH concentration takes place. GSH may be an important agent in maintaining structural integrity of the red cell, as may be TPNH, but the ultimate way in which glucose 6-phosphate dehydrogenase deficiency is linked to red cell drug susceptibility is unknown.

5 Drug-sensitive red cells can be detected *in vitro* by assaying them for glucose 6-phosphate dehydrogenase activity. They can also be detected by incubating red cells under standardized conditions with acetylphenylhydrazine and by measuring the reduced glutathione content, or by observing the pattern of Heinz body formation.

6 The red cell abnormality of drug sensitivity is an inherited, sex-linked dominant trait. Marked variability in the degree of penetrance is observed among affected females who are heterozygotes. The defect is widely distributed; it is common among American Negroes, Sephardic Jews, and Sardinians.

7 While red cells from patients with this defect have normal life spans, unless challenged by drug, a similar biochemical abnormality has also been described in a few patients with non-spherocytic hemolytic anemia.

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## Chapter 33

### The Hereditary Methemoglobinemias\*

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#### DEFINITION

Hereditary methemoglobinemia or hereditary methemoglobinemic cyanosis is a rare condition. More than one hundred cases have been reported in the literature. The disease has been described in Caucasians, Eskimos, and Chinese. Many of the patients have been of Greek stock.

The major feature of the disorder is a diffuse persistent slate gray cyanosis which is present from early infancy and unassociated with clubbing of the fingers or significant cardiac murmurs. Methemoglobin comprises 20 to 50 per cent of the total hemoglobin. Compensatory polycythemia and associated reticulocytosis are occasionally observed. The disease is frequently well tolerated and patients may reach an advanced age. Cardiac and respiratory distress are conspicuously absent although exertion may cause dyspnea, palpitation, easy fatigability, and severe headache. The conjunctival and retinal veins may be congested.

The blood has a chocolate hue and fails to turn a normal red color upon oxygenation. This is attributable to the presence in the erythrocytes of excessive amounts of methemoglobin (ferrihemoglobin), an oxidation product of hemoglobin.

The increased concentration of methemoglobin in this disorder occurs in the absence of any exogenous methemoglobin-producing substances. This serves to distinguish this syndrome from the acquired methemoglobinemias. Since normal individuals have small but definite amounts of methemoglobin in their blood, it is apparent that the formation of methemoglobin is a normal process. Accordingly, it must be assumed that there is a physiologic reducing system which can reconvert methemoglobin to hemoglobin.

The experimental work included in this report was completed while the author was a Research Fellow in the Department of Pediatrics, Harvard Medical School and the Department of Medicine, Children's Medical Center, Boston, Mass.

bin to hemoglobin. The existence of abnormal levels of methemoglobin indicates an imbalance between the oxidative and reductive processes.

### HISTORICAL INTRODUCTION

The first reports of cyanosis due to an abnormal blood pigment (presumably methemoglobin) stressed a supposed relationship with gastrointestinal disturbances in the descriptive phrase "enterogenous cyanosis" [1]. It was not until 1930 when Jitarczek et al. described an adult with cyanosis from early childhood [2] that the possibility of constitutional metabolic disorder was seriously entertained. The familial nature of the syndrome was suggested by Hitzemberger [3] and later confirmed by Bensley et al. in 1938 [4]. The latter authors in their report surveyed the world literature on hereditary methemoglobinemia and found only four sporadic cases of the idiopathic (metabolic) type in addition to the two reports of familial cases then available. Probably as a result of the decline in usage of methemoglobin producing drugs and of the increased interest in metabolic disease in general, a remarkable number of new cases of the idiopathic type has been recorded since their survey. More than 30 pedigrees are known which involve at least two affected members and a comparable number of isolated sporadic cases can be listed.

The major efforts to elucidate the fundamental mechanism of this disease have been chiefly those of the English worker Gibson. His studies of affected individuals from several families have demonstrated a defect in the enzyme system which is normally responsible for maintaining the hemoglobin in the reduced state [5]. This defect appears to be transmitted recessively.

Quite recently it has become apparent that Gibson's explanation is inapplicable to many of the reported cases. The first note of discrepancy was introduced by the studies of Hörlein and Weber of a German family in which the disorder could be attributed to a dominantly transmitted abnormality in the hemoglobin itself [6]. The dominant inheritance of congenital methemoglobinemia, particularly in Greek populations, has been stressed by Codrains [7]. A number of North American families also demonstrating dominant transmission have now been studied and in all an abnormal hemoglobin has been found with properties quite sufficient to explain the methemoglobinemia (see below) [8]. This group of abnormal hemoglobins is known as the Hb M type pigments, after the example of Singer [9] who applied the designation Hb M to the hemoglobin discovered by Hörlein and Weber [6]. The pigments are all characterized by the anomalous nature of their methemoglobin as indicated by the abnormal absorption spectrum of the derivative.

From the foregoing it is evident that hereditary methemoglobinemia is not a single disease but rather a group of diseases. On the basis of

current information three categories of differing etiology may be postulated (1) formation of an abnormal methemoglobin resistant to reduction, (2) a deficiency in the erythrocytic reducing system, with accumulation of "normal" methemoglobin, and (3) abnormal endogenous formation of oxidizing substances, with increased production of methemoglobin beyond the capacity of the system which normally maintains the hemoglobin in the reduced state.

## CHEMISTRY OF METHHEMOGLOBIN

### "Normal" Methemoglobin

Methemoglobin is the oxidized derivative of hemoglobin in which the iron of the four heme groups is changed from the usual ferrous to the ferric state. As a consequence the iron is no longer able to complex with oxygen. Methemoglobin is accordingly unable to serve as a respiratory pigment. These statements apply with equal validity to the methemoglobin derived from all known hemoglobins including the abnormal human hemoglobins and the several molecular species that comprise "normal" hemoglobin.<sup>1</sup>

As is true of heme pigments in general, methemoglobin has a characteristic spectral absorption curve. Employed in identification and measurement, its use rests upon the implicit assumption that the methemoglobins prepared from different hemoglobins are identical or very nearly so in their spectral properties. This assumption is quite valid for the various components of normal human hemoglobin and for most abnormal hemoglobins, but the Hb M-type pigments must be excepted. One should be aware that the absorption curve of methemoglobin changes with hydrogen ion concentration. That observed at pH 7.0 and below corresponds to the acidic form of methemoglobin, and that at higher pH values to alkaline methemoglobin.

Methemoglobin takes part in two major categories of reactions which are of particular interest here. It may be reduced to hemoglobin which can then combine with oxygen (to form oxyhemoglobin) or it may complex directly with a number of substances, especially nitrogenous compounds such as cyanide. In either case the spectrum is altered to an absorption pattern characteristic of the resulting compound. Under usual conditions these reactions affect equally all four heme groups of the molecule and are accompanied by a change in the total charge of the molecule. In the case of cyanmethemoglobin the addition of the cyanide ion neutralizes one positive charge of each heme group (since one univalent anion combines with each ferric atom) and the net charge is equal to that of oxyhemoglobin.<sup>2</sup>

<sup>1</sup> The heterogeneity of normal hemoglobin is discussed in detail in the section on the hemoglobinopathies (see Chap. 34).

<sup>2</sup> Heme-linked ionizations are not considered in this discussion.

*Gasometric Methods of Analysis*

The loss of oxygen-combining power may be used as a measure of the amount of methemoglobin by comparing the total oxygen capacity with that expected from the total amount of heme pigments present. The latter is separately determined by estimation of the heme groups (as by iron analysis or as acid hematin). This method usually overestimates the concentration of methemoglobin since of necessity the result also includes other non-oxygen-combining pigments such as sulfhemoglobin. This technique indicates that not more than 2 per cent of the hemoglobin of normal blood can be in the methemoglobin form [10].

*Spectroscopic Methods of Analysis*

In essence all spectroscopic methods assay the amount of methemoglobin by quantitating the spectral change when methemoglobin is chemically altered either by complex formation or by reduction. The most commonly used technique is that of Evelyn and Malloy [11]. The methemoglobin is reacted with cyanide and the change in light absorption at 632 m $\mu$  is measured. This method is quite specific in the absence of the Hb M-type pigments. The normal concentration of methemoglobin obtained with cyanide techniques is less than 2 per cent of the total pigment [12].

Since Hb M-type pigments in the methemoglobin form have abnormal absorption spectrums the usual quantitative spectroscopic tests give erroneous values for the degree of methemoglobinemia in Hb M disease. With certain varieties of Hb M disease the circulating pigment does not react with cyanide and a negative result for methemoglobin is obtained in spite of the obvious cyanosis of the patient. Qualitative spectroscopic tests which depend upon identification of the characteristic absorption band (at 632 m $\mu$ ) will likewise not give the expected finding. In many cases of Hb M disease no abnormal absorption band will be seen with the hand spectroscope.

## HEREDITARY METHEMOGLOBINEMIA ASSOCIATED WITH THE SPECTROSCOPICALLY ABNORMAL METHEMOGLOBINS (THE Hb M DISORDERS)

*Electrophoretic and Spectrochemical Behavior of Normal Hemolyzates*

Since the isolation of the Hb M-type pigments has been accomplished only very recently the information concerning them is relatively limited and is confined largely to the electrophoretic and spectroscopic behavior of certain derivatives. A few preliminary remarks concerning the electrophoretic behavior of hemolyzates prepared from the blood of normal individuals is a necessary introduction to this subject (a more extensive discussion will be found in the section on the hemoglobinopathies Chap

34) By utilizing various techniques, the hemoglobin of normal hemolyzates can be shown to contain a minimum of three distinct components (Hb A, Hb A<sub>2</sub>, and Hb F). These probably differ considerably in their intimate chemical structure. Under the conditions used in the study of the Hb M-type pigments (electrophoresis on starch block at pH 7.0) normal hemolyzate migrates as a single band toward the cathode. Normal hemolyzate also migrates as a single band (toward the cathode) after oxidation to methemoglobin, although now the rate of migration is more rapid because of the increase in positive charge. After neutralization of the charge by treatment with cyanide, the pigment (cyanmethemoglobin) migrates as a single band with essentially the same mobility as oxyhemoglobin.

#### *Electrophoretic and Spectrochemical Behavior of the Hb M-Type Pigments*

In the blood from all individuals exhibiting methemoglobinemia of the dominantly transmitted type so far examined, electrophoresis of

TABLE 33-1 SPECTROSCOPIC AND CHEMICAL PROPERTIES OF THE Hb M-TYPE PIGMENTS (IN M/15 SODIUM PHOSPHATE BUFFER, pH 6.5)

| Pigment              | Maxima in visible region $m\mu$ | Gross color of electrophoretic band | Cyanmethemoglobin spectrum† |
|----------------------|---------------------------------|-------------------------------------|-----------------------------|
| Methb A              | 502-632                         | Brown                               | Normal                      |
| Methb M <sub>B</sub> | 495-602                         | Gray                                | Abnormal                    |
| Methb M <sub>B</sub> | 492-602                         | Green                               | Normal                      |
| Methb M <sub>M</sub> | 500-602                         | Gray-green                          | Normal                      |

Hb M Boston type (Hb M<sub>B</sub>) was isolated from the family reported by Gerald et al [13]. Hb M Saskatoon type (Hb M<sub>S</sub>) from that of Baltzan and Sugarman [14] and Hb M Minneapolis type (Hb M<sub>M</sub>) from that of Pesciotta and Hinz [15].

† Those pigments with an abnormal cyanmethemoglobin spectrum are not associated with increased concentration of methemoglobin in the patient when the methemoglobin is determined by its reaction with cyanide.

hemolyzates under appropriate conditions has disclosed an abnormal pigment. These pigments collectively known as the Hb M-type pigments are anomalous in that the relative electrophoretic mobilities of the oxyhemoglobin, methemoglobin, and cyanmethemoglobin derivatives differ from that predicted by experience with normal hemoglobin (Fig. 33-1). The untreated hemolyzate migrates as a single band. After conversion of all the pigment to methemoglobin by treatment with ferricyanide, two distinct bands are observed. After conversion to cyanmethemoglobin, a single band is again observed which has a mobility comparable to that of the initial hemolyzate. These findings are incompatible with the charge differences among oxyhemoglobin, methemoglobin, and cyanmethemo-

globin derivatives as previously described, and are best explained by assuming that the increase in positive charge of the methemoglobin, over that present in the oxyhemoglobin and cyanmethemoglobin forms is less for the Hb M-type pigments than for normal hemoglobin. Very little or no charge difference apparently exists between the normal and the

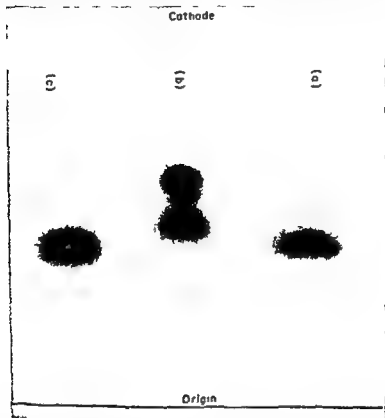


Fig. 33-1. Electrophoretic behavior on starch block of a hemolyzate containing both Hb M and Hb A: (a) Untreated hemolyzate; (b) after conversion to methemoglobin; (c) after conversion to cyanmethemoglobin. (Electrophoresis in sodium phosphate buffer pH 7.0; ionic strength 0.10.)

abnormal fractions of the hemolyzate when present as oxyhemoglobin or cyanmethemoglobin—a significant difference appears only when the methemoglobins are compared.

The electrophoretically purified Hb M-type pigments have been studied spectroscopically and are most unusual [8]. The absorption maxima of the acidic methemoglobin form of three different Hb M-type pigments are given in Table 33-1. These are designated Hb M<sub>1</sub>, Hb M<sub>2</sub>,



instance the usual methemoglobin band could not be demonstrated in a patient who was intensely cyanotic [20]. Finally, the pedigree of sulfhemoglobinemia described by Miller [26] and that of idiopathic cyanosis by Lutembacher [27] cannot be distinguished from instances of Hb M<sub>s</sub> disease by the tests reported.

### *Erythrocyte Metabolism in the Hb M Diseases*

The limited studies performed to date have failed to demonstrate any abnormality of the erythrocyte enzyme systems. The rate of reduction of methemoglobin with cells treated *in vitro* with oxidizing agents has not differed significantly from that observed with normal red cells even under the conditions which successfully demonstrate the defect in the enzymatic variety of hereditary methemoglobinemia [28]. Pisciotta et al. [16] observed in one patient<sup>4</sup> that the concentration of methemoglobin in freshly shed blood did not change after incubation of the cells with fresh normal plasma or with glucose. Also if the concentration of methemoglobin in the cells was first increased to 90 per cent (from the *in vivo* level of 30 per cent) by treatment of the cells with nitrate incubation with fresh normal plasma resulted in a decrease of methemoglobin to the original 30 per cent value in a few hours. This result is in accord with a normal enzyme system which can convert normal methemoglobin to the reduced state but which is less able to cope with abnormally reactive oxidized hemes of the Hb M-type pigments.

The life span of the erythrocyte as determined by reticulocyte count and serum bilirubin is normal in the Hb M diseases. The one reported exception to this was in a patient possessing both a Hb M-type pigment and an additional unidentified (Hb C like) hemoglobin [21].

### *Clinical Physiology*

On the basis of the proposed theory it is reasonable to expect that the methemoglobin formed *in vivo* in Hb M diseases would be derived almost solely from abnormally reactive hemes of the Hb M-type pigments. The failure to detect abnormal blood concentrations of methemoglobin by the Evelyn and Malloy test in two families is consistent with this [13-21]. As a result there is an upper limit to the impairment in oxygen combining power which on this basis should not exceed one-half the concentration of the Hb M-type pigment. The abnormal reactivity is also responsible for the resistance to ascorbic acid and methylene blue therapy seen in some cases of Hb M disease [13-21].

A Hb M-type pigment (Hb M<sub>s</sub>) has since been isolated by the author from the blood of this patient samples of which were generously made available by Dr. Pisciotta.

*Genetics*

Since the abnormality in the Hb M-type pigments is limited to the globin of the molecule the e hemoglobins may be classed with the more common abnormal hemoglobins (see Chap 34). Like them the Hb M-type pigments have been transmitted as a codominant character involving both sexes in all the pedigrees adequately studied. No abnormality has been commonly associated with the abnormal hemoglobin except the e secondary to its presence. All reported cases have come from European and Mediterranean populations.

### HEREDITARY METHEMOGLOBINEMIA ASSOCIATED WITH A DEFICIENCY OF THE METHEMOGLOBIN REDUCING SYSTEM

#### *Methemoglobin Reduction in the Normal Human Erythrocyte*

It is assumed that the small but definite amount of methemoglobin in normal blood represents an equilibrium between the processes causing methemoglobin formation and those accomplishing its removal. Except for the effect of oxygen little information is available concerning the factors responsible for the normal formation of methemoglobin; hence the following remarks will be confined to the methemoglobin reducing process. Since there is a significant interspecies variation in these reactions the discussion will be confined to results obtained in studies of human erythrocytes.

The reduction of methemoglobin in the normal human red cell proceeds slowly in a saline suspension but is increased by the addition of glucose to the medium and nearly equally so by the addition of lactate [29]. With either substrate the reduction is accompanied by the formation of an equivalent amount of pyruvate [29] (see Fig 33-2 for a condensed outline of the glycolytic pathway). Methemoglobin reduction is probably linked to glycolysis through reduced diphosphopyridine nucleotide (DPNH) [29]. Evidence for this is the inhibitory action of iodoacetate on methemoglobin reduction when glucose is the substrate and its lack of effect when the glucose is replaced by lactate (see Fig 33-2). In keeping with this scheme is the failure of fluoride to affect reduction in the presence of glucose [29].

Certain autooxidizable dyes such as methylene blue (MB) are capable of accelerating the reduction of methemoglobin in the presence of the proper substrate. When MB is added along with glucose a tenfold increase is obtained. The amount of pyruvate (or of phosphoglycerate when fluoride is present) formed under these conditions is considerably less than that expected from the amount of methemoglobin reduced in contrast to the results obtained without MB. The MB thus appears to

activate a metabolic pathway which is not operative in its absence [20] From studies of normal and hereditary methemoglobinemic erythrocytes, Gibson concluded that this pathway was the hexose monophosphate shunt [20] the initial and pertinent steps of which are given in Fig 33-3

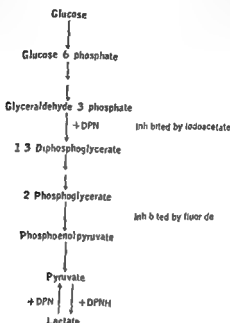


Fig 33-3 Condensed outline of the glycolytic pathway [30] The omission of intermediate reactions is indicated by a double arrow

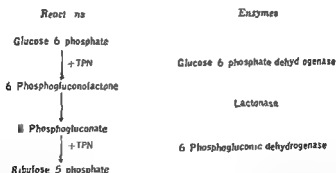


Fig 33-3 Initial reactions of the hexose monophosphate shunt [31]

It should be noted that TPNH rather than DPNH is produced by this reaction. Indirect evidence supporting Gibson's contention is now available from the studies of cells deficient in glucose 6 phosphate dehydrogenase (C 6 I D). Red cells deficient in C 6-I D activity are concomitantly defective in reducing methemoglobin in the presence of glucose plus MB [32-33].

The majority of investigators though by no means all believe that the reaction between methemoglobin and reduced pyridine nucleotides is an enzymatically controlled step. Kiese obtained direct evidence in favor of this by preparing an extract from horse erythrocytes which was capable of reducing methemoglobin in the presence of TPNH [34]. His term for the enzyme *methemoglobin reductase* has since been adopted in the current literature for the human counterpart. Gibson assumed the existence of such an enzyme but postulated that two comparable systems were present, one active toward TPNH and the other toward DPNH [29]. Recently Huennekens et al isolated an enzyme (which they called methemoglobin reductase) from human erythrocytes which catalyzed the reaction of methemoglobin with TPNH [35]. Unexpectedly this preparation had a significant though lesser (one-fifth as great) activity with DPNH.

A preliminary account is now available of an investigation furnishing direct evidence for the presence in normal human erythrocytes of an enzyme capable of reducing methemoglobin which has a DPNH preference [36]. In this and in other properties it differs significantly from the methemoglobin reductase of Huennekens et al. Its probable importance in the reduction of methemoglobin is indicated by the observation that this particular enzyme is deficient in the erythrocytes from the few patients with hereditary methemoglobinemia (of the recessively transmitted type) so far studied.

#### *Methemoglobin Reduction in Erythrocytes from Patients with Enzymatic Hereditary Methemoglobinemia*

It has long been suspected that the abnormality in hereditary methemoglobinemia is an intrinsic disorder of the erythrocyte. Lian et al found that methemoglobin has little or no tendency in vitro to disappear spontaneously from hereditary methemoglobinemic cells [37] in contrast to the rapid reduction observed with cells from toxic methemoglobinemia. A more dramatic and elaborate confirmation of this clinical conclusion was obtained by Eder et al who transfused cells from a congenitally methemoglobinemic individual into a patient with aplastic anemia and demonstrated the persistence of methemoglobin within the erythrocytes despite their being in a foreign circulation [38].

Incubation of erythrocytes from patients with hereditary methemoglobinemia of the enzymatic type in saline containing glucose is not associated with the rapid reduction of the methemoglobin which is seen with normal cells in which methemoglobin has been produced by chemical treatment. This is observed in spite of apparent adequacy of the glycolytic enzyme systems in such cells [29]. Preservation of glycolysis is inferred from the rapid reduction of pyruvate to lactate in the presence of glucose plus fluoride as in normal cells. However in the presence of both MB and glucose, normal and hereditary methemoglobinemic cells are equally

activate a metabolic pathway which is not operative in its absence [29]. From studies of normal and hereditary methemoglobinemic erythrocytes Gibson concluded that this pathway was the hexose monophosphate shunt [29], the initial and pertinent steps of which are given in Fig. 33-3.

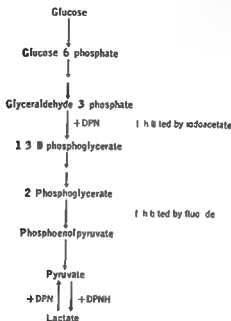


Fig. 33-2 Condensed outline of the glycolytic pathway [30]. The omission of intermediate reactions is indicated by a double arrow.

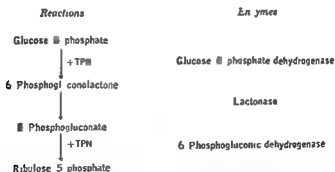


Fig. 33-3 Initial reactions of the hexose monophosphate shunt [31].

It should be noted that TPNH rather than DPNH, is produced by this reaction. Indirect evidence supporting Gibson's contention is now available from the studies of cells deficient in glucose 6-phosphate dehydrogenase (G-6-PD). Red cells deficient in G-6-PD activity are concomitantly defective in reducing methemoglobin in the presence of glucose plus MB [32, 33].

definite shift to the left of the curve was observed [3 43-44] and in one instance a slight shift [2] but in two no alteration could be demonstrated [38 45] Gibson has emphasized the significance of this discrepancy and has suggested that absence of the alteration in the oxygen dissociation curve implies an uneven distribution of methemoglobin i.e. some erythrocytes contain only methemoglobin and the remainder only reduced (ferro) hemoglobin [5] It is of course possible that these are two groups of cases which differ basically in their etiology

### *Clinical Physiology and Therapy*

The defect in oxygen carrying capacity of the blood of the patients has not been sufficient to cause symptoms referable to oxygen deficiency except in a few cases nor has there been a direct effect upon the survival of the red cell

The therapy of this disease is based upon the promotion of *in vivo* reduction of the methemoglobin by administration of MB or ascorbic acid Although ascorbic acid is nearly as effective as MB its mode of action is still but poorly understood MB administered intravenously causes an abrupt fall of the methemoglobin concentration to near normal values while ascorbic acid administration is associated with a gradual decline to a steady level which is still somewhat above normal [45] Alleviation of the cyanosis with either of these agents is obtained only so long as therapy is continued

The intensity of cyanosis varies with the diet and with the season which may possibly be a reflection of ascorbic acid intake The onset of cyanosis is variable In a few cases it has been observed immediately or a few hours after birth [46] In others it has not been noticed until adolescence [4] Whether this is related to diet or to exposure to methemoglobin promoting agents is not known

Although the appearance of cyanosis in early infancy suggests the very real possibility of hypoxic brain damage this complication has been reported relatively infrequently [3 46-49] The occurrence of mental deficiency in all the methemoglobinemic children (total of six affected) of two pedigrees [47, 48] suggests a unique difference in the etiology of their methemoglobinemia

### *Genetics*

Until recently information on the inheritance of hereditary methemoglobinemia was confused by pedigrees documenting both recessive and dominant transmission Some of the families with dominantly inherited cyanosis are now known to be affected with one of the Hb M diseases and in many of the remaining families indirect evidence suggests that they also belong to this same category On the basis of present information it seems reasonable to anticipate that the two inheritance patterns will

able to reduce methemoglobin. It was this contrasting behavior which led Gibson to postulate that MB activates an otherwise inactive reduction pathway [29]. On the assumption that reduction of methemoglobin (in the absence of MB) by DPNH is controlled by a specific DPNH dependent enzyme, Gibson considered that a deficiency of this enzyme was the basis of the defect in hereditary methemoglobinemia [29]. Gibson developed an assay method supposedly specific for this erythrocytic enzyme and was able to demonstrate a marked deficiency in five methemoglobinemic patients and a slight deficiency in two others [29-39].

As previously mentioned, further evidence is now available to corroborate Gibson's hypothesis that methemoglobin reduction is normally controlled by a DPNH-dependent enzyme [36]. The direct demonstration of this enzymatic activity was made possible by the development of an improved assay system. Scott and Griffith have shown that the reaction between 2,6-dichlorobenzene indophenol and DPNH is catalyzed by an enzyme present in the hemolysates from normal individuals. The hemolysates from patients with hereditary methemoglobinemia, however, failed to exhibit such activity [40].

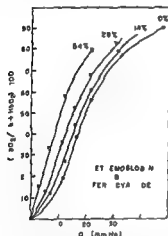


Fig 33-4 The effect of methemoglobin as formed by ferricyanide on the oxygen dissociation curve of human hemoglobin. 0.6 M phosphate buffer, pH 7.4. With increasing methemoglobin the dissociation curve shifts to the left [41].

### The Oxygen Dissociation Curve in Hereditary Methemoglobinemia

Methemoglobin is unable to bind oxygen because of the altered state of the iron. In addition, there is an indirect effect resulting from the interaction of the four heme groups in the hemoglobin molecule. Oxidation of one of the heme groups in a given

molecule affects the tenacity of the binding with oxygen of the remaining unoxidized groups. This is one manifestation of a more general phenomenon known as heme-heme interaction. This is most clearly demonstrated by plotting oxygen saturation of the hemoglobin against oxygen pressure of the gaseous phase—the oxygen dissociation curve. The heme-heme interaction theory predicts that following partial oxidation of the hemes, a change in shape of the curve and a shift to the left (greater saturation at lower oxygen tension) will occur. This prediction was confirmed by the experimental findings of Darling and Roughton with hemoglobin after partial oxidation *in vitro* [41] (Fig 33-4).

The oxygen dissociation curve in hereditary methemoglobinemia has been determined by seven different investigators. In four instances a

(b) a deficiency in the erythrocyte enzyme system which maintains hemoglobin in the unoxidized state and (c) abnormal endogenous formation of substances capable of oxidizing hemoglobin to methemoglobin. Although only a single possible case is available to illustrate the last category the first two pathogenetic mechanisms appear to be of nearly equal importance in the causation of hereditary methemoglobinemia.

3 The frequency of abnormal methemoglobins resistant to reduction has only recently been realized. These methemoglobins which are the oxidation products of a group of different hemoglobins categorically designated the Hb M-type pigments are unique in their electrophoretic and spectrochemical behavior. Their spectral absorption curves differ from that of methemoglobin prepared from normal human blood and hence from that of any other vertebrate methemoglobin. Consequently the usual spectroscopic and chemical tests for methemoglobin may not be applied to blood containing a Hb M-type pigment without risking quantitative or even qualitative error.

Since the molecular abnormality of the Hb M-type pigments lies in the globin these may properly be considered as further examples of the abnormal hemoglobins. As might be expected in pedigrees exhibiting this condition the cyanosis is transmitted as a dominant character.

4 The second major pathogenetic category of hereditary methemoglobinemia derives from a deficient intracerythrocytic enzyme system for reducing methemoglobin. Present evidence favors the theory that the disease arises from a deficiency of a diphosphopyridine nucleotide-dependent enzyme which is normally responsible for reducing methemoglobin.

5 Alternatively hereditary methemoglobinemia of the enzyme deficiency type may be a heterogeneous group of conditions. Of particular interest in this regard is the seemingly inextricable association of hereditary methemoglobinemia and mental defect in some families and the unexpected finding of oxygen dissociation curves without the shift to the left obtained in others.

6 The two main categories of hereditary methemoglobinemia differ not only in spectroscopic findings but also on genetic grounds. It seems probable that the pedigrees with dominant transmission of cyanosis represent instances due to a Hb M type pigment and that the recessive variety is due to erythrocytic enzyme deficiency.

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divide the cases into Hb M disease (dominant) and the enzyme deficiency variety (recessive)

When a rare disease is recessively inherited the proportion of pedigrees derived from consanguineous matings is usually appreciable. Surprisingly, only a single instance of consanguinity has been noted among the pedigrees of hereditary methemoglobinemia of the recessive type [49]

Like the Hb M diseases the enzyme deficiency variety has been described only in populations with a light complexion. This may reflect the difficulty of detecting cyanosis in a dark-skinned person. Both European and non-European peoples [40, 50] have been affected. No predilection for either sex has been noted.

### CONGENITAL METHEMOGLOBINEMIA DUE TO AN EXCESS OF ENDOGENOUS OXIDIZING SUBSTANCES

At present this category is largely hypothetical. One case has been reported which may fall within it. Fishberg has described a patient with idiopathic methemoglobinemia and severe cyanosis (no clinical details were given) who excreted large amounts of benzoquinoneacetic acid in the urine even while on a normal diet [51]. Methemoglobin concentrations as high as 20 per cent were observed during the period of benzoquinoneacetic acid excretion and the actual blood concentration of methemoglobin varied roughly with the output of this substance. Ascorbic acid administration rapidly abolished both the excretion of benzoquinoneacetic acid and the methemoglobinemia. It was concluded by Fishberg that the methemoglobinemia was the direct result of the oxidation of the hemoglobin by benzoquinoneacetic acid. Although the data are not adequate to determine whether the presence of the latter resulted from a metabolic disorder or from a transient ascorbic acid deficiency, the frequency of ascorbic acid deficiency without concomitant cyanosis suggests that a metabolic error was present in Fishberg's case.

### SUMMARY

1 Hereditary methemoglobinemia is characterized by chronic cyanosis associated with increased amounts of intravascular methemoglobin, the oxidation product of hemoglobin. Since small amounts of methemoglobin may be identified in normal blood, it is believed that an equilibrium exists between the processes responsible for oxidation of the hemoglobin to methemoglobin and those which facilitate the reverse reaction. Hereditary methemoglobinemia is the result of a hereditary imbalance in these two competing processes.

2 At present three different pathogenic mechanisms appear possible:  
(a) the formation of an abnormal methemoglobin resistant to reduction,

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## ADULT AND FETAL HEMOGLOBIN

About one-hundred years ago von Korbër [1] noticed that the blood of the adult is more easily denatured by alkali than that from the placenta. He concluded that there are two human hemoglobins—adult and fetal. These are now designated A and F. Hemoglobin F is present in infants at birth when it varies in concentration from approximately 60 to 90 per cent. It then gradually disappears from the circulation and by the age of 4 months only traces persist.

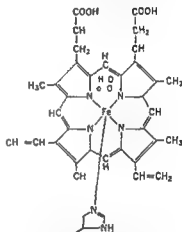
Jonxis [2] has pointed out that the presence of Hb F in the blood of normal children over 11 months old may be explained only by the synthesis of this pigment after birth. Formation of Hb F may persist in pathologic states which begin at an age when Hb I is still being produced and if the cause of the infantile disorder usually an anemia is not removed Hb F may still be found in adult life. This was first observed in thalassemia [3-6] but was later also found in sickle cell anemia [7] and other hemoglobinopathies. The phenomenon is not specifically associated with this group of disorders but can be observed in many other syndromes such as spherocytic jaundice, leukemia and nutritional anemias.

The numerous differences between adult and fetal hemoglobins have been summarized by White and Beaven [8] and by Bethe [9]. These include differences in solubility, ultraviolet spectrum and resistance to alkali denaturation. There may be differences also for oxygen affinity and in antigenic activity. Hemoglobin F is inherited independently of all the adult hemoglobin variants. These and other properties listed below divide it sharply from Hb A and its variants.

**Alkali Denaturation.** At pH 12.5 (0.04 N alkali) and at room temperature the half reaction time of denaturation ( $t_{1/2}$ ) for Hb A is 10 sec. and for Hb F is 1,000 sec.

**Ultraviolet Spectrum.** The tryptophan fine-spectrum band for Hb A is 291.0  $m\mu$  and for Hb F is 299.8  $m\mu$ .

**Electrophoresis.** The isoelectric point of Hb A is 6.87 and of Hb F is 6.95. Upon bouillon electrophoresis at pH 6.5 A and F separate. On paper electrophoresis at pH 8.0 A and F do not separate but when present in a mixture F causes lowering of the A band. Upon starch gel electrophoresis at pH 8.6 Hb F separates as a distinct band behind Hb A. With agar electro-



Histidine residue of globin

FIG. 34.1. Heme, one of the four identical prosthetic groups attached to one molecule of globin.

## Chapter 34

### The Hemoglobinopathies and Thalassemia

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*Hermann Lehmann and J. A. M. Ager*

The observations of Pauling and his collaborators on the electrophoretic characteristics of human hemoglobins first published in 1949 form a landmark in biochemical genetics. Their observations and many which have followed have firmly established the existence of various species of human hemoglobin and have shown that a subject may have various combinations of these species circulating in his blood at the same time. The inheritance of the hemoglobins follows strict genetic patterns and certain dyscrasias of the blood may quite precisely reflect the genetic pattern of the constituent hemoglobins.

This chapter will include a review of present information on the chemical nature of the hemoglobins and the genetics of their inheritance and will attempt to classify the variant hemoglobins. In addition the diseases arising from an endowment of abnormal hemoglobin will be described and interpreted and present information regarding the nature of thalassemia discussed.

#### HUMAN HEMOGLOBIN

Hemoglobin is the principal protein of man concerned with oxygen transport. Each molecule of hemoglobin consists of one molecule of globin to which are attached four molecules of ferriprotoporphyrin IX (Fig. 34-1). This prosthetic group is common to groups of compounds concerned in redox processes in both the plant and animal kingdoms from the peroxidase of horse radish to the cytochromes of man. The protein part of the hemoglobin molecule shows a wide variation of molecular weight in the lower animals but in the vertebrates the molecular weight is relatively constant at about 68 000 (66 700 for man).

component, Hb A<sub>1</sub>, and a minor component amounting to about 3 per cent Hb A<sub>2</sub> [12]

Hemoglobin A<sub>2</sub> is a definite separate entity. It is noteworthy that it is found in the absence of Hb A<sub>1</sub> in sickle cell homozygotes. Also like Hb F it is increased in thalassemia in which Hb A<sub>1</sub> formation is decreased. Occasionally Hb A<sub>2</sub> separates into two fractions on electrophoresis A<sub>2</sub> and A<sub>2</sub>' [13-14] (Fig. 34-2). The trait A<sub>2</sub> + A<sub>2</sub>' segregates as a simple Mendelian alternative to single A<sub>2</sub>. Matings of persons with A<sub>2</sub> and A<sub>2</sub>A<sub>2</sub>' produce both types in equal proportions in the offspring.

A minute fraction on the leading edge of the A<sub>1</sub> band may also appear upon electrophoresis of normal hemoglobin. This small pointed area of hemoglobin has been designated Hb A<sub>3</sub>. Its significance is not yet clear nor is that of a small fraction of hemoglobin which may also be seen in front of the A band on chromatography at pH 6. As will be discussed later the two  $\alpha$  and two  $\beta$  polypeptide chains which together form the globin molecule differ in electrophoretic and chromatographic properties. Dissociation and recombination of these chains might take place in unequal proportions particularly at slightly acid pH. This could cause the appearance of small fractions differing from A<sub>1</sub> on electrophoresis and chromatography.

#### DIFFERENT ADULT HEMOGLOBINS

Until a few years ago interest in adult hemoglobin was centered on its prosthetic group. The heme. Carboxyhemoglobinemia, sulfhemoglobinemia, and methemoglobinemia were of clinical as well as of theoretic importance and it was known that methemoglobinemia could be either acquired or inherited. The inherited type was attributed to abnormalities in reducing systems which normally counteract the tendency toward methemoglobin formation in the red cells (cf. Chap. 33). Hörlein and Weber [15] studied a family in which the condition appeared to be associated with the hemoglobin molecule. For when globin and heme of the affected members of this family were separated and combined with normal heme and globin respectively, the abnormality went with the globin part of the respiratory pigment. Watson and her collaborators [16] had noted that the sickling phenomenon was only slight in infants and became more pronounced later when the fetal variant had been replaced by adult hemoglobin. It was the discovery of Pauling and his collaborators [17] of a second adult human hemoglobin in sickle-cell blood that became a milestone along the road of twentieth century investigative medicine. From that discovery has flowed an abundance of new light on the chemistry of human proteins, the physiology of hemoglobin, and the nature of numerous disease states of the blood. Few single papers have been the beginning of so many lines of investigation, many of them of far-reaching consequence.

chromatography' at pH 6.2 Hb F separates as a distinct band in front of Hb A.

*Chromatography* Hemoglobin F separates as a distinct band in front of Hb A on Amberlite IRC 50 at pH 8 or on carboxymethylcellulose.

*Solubility* Reduced Hb I is more soluble in phosphate buffer than is reduced Hb A.

*Amino Acid Composition* There are numerous differences. The most notable is in isoleucine content which is absent from pure Hb A and is 1.53 per cent in Hb F. The four terminal residues in Hb A are valyl only but in Hb F there are two valyl and two glycyl residues.

*Immunology* Antisera have been prepared from chickens, rabbits and other species which precipitate specifically Hb A and F respectively. Agglutinating sera have been prepared from rabbits which act on the red cells of the newborn but not on those of the adult except when the adult blood contains Hb F.

*Oxygen Dissociation* The oxygen affinity of cord blood is greater than that of adult blood. The difference disappears after dialysis against phosphate buffer.

#### HETEROGENEITY OF NORMAL ADULT HEMOGLOBIN [4-7]

Hemoglobin A is not a homogeneous protein. Derrien [10] has summarized his own experiences and that of other workers with fractional

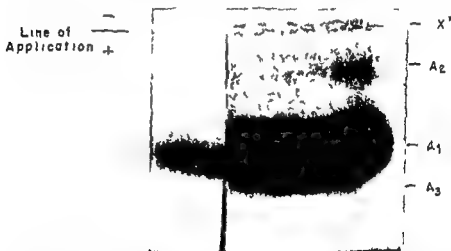


Fig. 34.2 The heterogeneity of hemoglobin. When normal adult hemoglobin is examined by paper electrophoresis at pH 8.6 a single fraction is observed (see left unstained strip). On staining several additional minor fractions appear. These are Hb A<sub>1</sub> and A<sub>2</sub> and a nonhemoglobin component.

"salting out" of Hb A. He found six different components. Itano [11] considers these results due to the transition of soluble protein into the solid phase where the crystal forms differ at different salt concentrations. There can be no doubt that adult hemoglobin consists of at least two fractions which can easily be separated by electrophoresis, a major

gested in 1947 [26] that there might be an alternative hypothesis similar to that which had differentiated between severe and mild thalassemia in terms of their being homozygous and heterozygous states. In 1949 the same theory together with supporting evidence was put forward by Beet [27] and Neel presented conclusive proof based on numerous family studies [28].

The discovery of the sickle cell hemoglobin correlated all the data that had been accumulating. Sickling was due to an abnormal hemoglobin

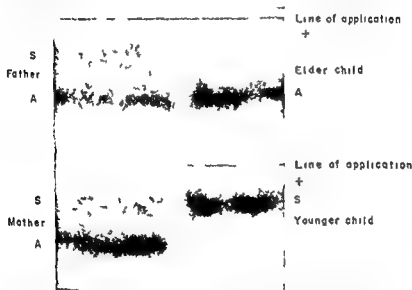


Fig 34-4 The electrophoretic pattern of a sample of blood obtained from two of three of parents each of whom was a sickle-cell trait carrier. Run at pH 8.6. On the left above a sample from the father below one from the mother. On the right are samples from the elder child and the younger child.

and whereas in the heterozygous carriers of the sickle-cell trait both Hb A and Hb S were found, no A was present in patients with sickle cell anemia who were homozygous for the abnormal gene. Thus the gene for Hb A found expression in the phenotype in the presence of another for Hb S (Fig 34-4). Another hemoglobin besides S was found in small amounts in the blood of patients homozygous for sickling, but this was not Hb A. It was later shown to be Hb F persisting in these patients beyond the age of infancy [7].

Almost all the findings in sickle-cell disease can be explained by the physical properties of reduced Hb S and particularly by its low solubility. Harris [4] found that the deoxygenation of concentrated solutions of Hb S results in a semi solid gel. Under the microscope tactoids 1 to 15  $\mu$  long may be observed which are remarkably similar in shape to sickled



*Sickle cell Hemoglobin (Hemoglobin S)*

In 1910 Herrick [18] examined the blood of a severely anemic West Indian Negro student residing in Chicago and found it to contain "peculiar elongated and sickle-shaped red corpuscles." It was soon found that some Negroes had erythrocytes which appeared to be perfectly normal *in vivo*, but which could be induced to take on the sickle shape *in vitro*. Hahn and Gillespie [19] reported that sickling occurs only with low oxygen tensions and that sickled cells can revert to the normal shape



Fig. 34.3 Sick cells from the peripheral blood as they appear under low oxygen tension.

when they are exposed to sufficient oxygen (Fig. 34.3). Diggs et al. [20] distinguished between those individuals in whom the sickling of the red cells was associated with a profound disease, sickle-cell anemia, and others in whom the abnormality was a harmless trait. Sherman [31] showed that the two conditions also differ in their tendency to sickle on deoxygenation. Later it was seen that the first type of cell has a shorter life span than normal cells and that the trait cells have a normal survival time even when transfused into patients with sickle cell anemia [22, 23].

The sickling phenomenon is familial and the mode of inheritance is that of a single Mendelian dominant [24, 25]. Expression of a gene in this way in the heterozygote is now often referred to as codominance. At first it was generally assumed that the same gene produces in some persons an asymptomatic condition, the sickling trait, and in others is responsible for the severe form with anemia and other changes. Neel sug-

of two other abnormal hemoglobins. When genes for Hb A and Hb S are inherited together both hemoglobins are incorporated into the same cells. This can be concluded because in sickle cell preparations from trait carriers all cells take on the abnormal shape. However the proportion of



Fig 34.5 X-ray appearance of the skull of an adolescent patient with sickle-cell anemia. Note the thickened diploë and virtual absence of an outer table. There appears to be a ring infarct in the frontal region. (By permission of Dr W P Cockshull, University College Hospital, Ibadan, Nigeria.)

the two hemoglobins is unequal: only 20 to 40 per cent is Hb S. Accordingly the manufacture of S must proceed more slowly. That Hb A production prevails over that of S explains why sickle-cell heterozygotes carry the abnormal hemoglobin but do not suffer from the anemia.

### *Hemoglobin C*

The observation of atypical cases of sickle-cell disease with failure to find sickling in one of the two parents led to the discovery by Neel and his colleagues of a new second abnormal hemoglobin in the nonsickling parent and to the demonstration of the same pigment in the children with the intermediate type of sickle-cell disease [37-38]. In these patients hemoglobin S amounts to more than half the total hemoglobin; the con-

cells. On reoxygenation the tactoids disappear but they form again when the oxygen is removed. Perutz and his collaborators [30, 31] reported that reduced Hb S is much less soluble than reduced Hb A. Oxygenated Hb A and Hb S have the same solubility, but upon deoxygenation the solubility of Hb A falls by one half whereas that of the sickle cell hemoglobin becomes fifty times less [11]. Sickling may therefore be understood as the outcome of intracellular tactoid formation in reduced sickle cells. The greater the concentration of the reduced Hb S the more it is likely to form tactoids, and accordingly the tendency to sickle is more pronounced in sickle cell anemia than in sickle trait. The gelling of hemoglobin inside the cells increases blood viscosity. Indeed virtually all the features of the disease can be related directly or indirectly to the increase in blood viscosity which accompanies deoxygenation [32, 33]. The slower the blood flow the greater will be the deoxygenation and the more intense will be the transformation of the discoid red cell into the spoked sickled cell. These abnormally shaped cells will be phagocytized by reticuloendothelial cells; this may explain why sickle cells have a shorter life span. Should the viscous bizarre shaped cells cause blockage of small vessels a vicious circle is set up. Deoxygenation becomes more intense and the cell shape becomes more and more abnormal.

The time factor is important in the development of sickling [34]. With a normal circulation time sickle cells remain in the deoxygenated state less than the 15 sec required for sickling to begin but if for any reason they are held up in organs with a low oxygen tension there will be time for sickling. A few sickled cells are invariably found in the circulating blood in the anemia.

The clogging of small blood vessels is responsible for the "crises" of sickle cell anemia. Infarctions which can occur in any part of the body may appear as acute abdominal emergencies, pneumonia, priapism, heart disease or hematuria. Necrotic areas in the long bones arising from the closure of nutrient vessels tend to become infected and osteomyelitis is a frequent complication. A *Salmonella* osteomyelitis is almost pathognomonic of sickle cell disease. The curious x-ray appearance of the skull in Hb S disease is not fully explained (Fig. 34-5).

On the whole and for practical purposes one can assume that the sickle cell trait is harmless. However splenic infarction can occur when sickle cell trait carriers fly at high altitude in unpressurized aircraft. Severe otherwise unexplained hematuria has been seen not infrequently in patients whose only abnormality was found to be a sickle cell trait [35, 36].

The recognition of Hb S, the studies of its biochemical and physical properties and the examination of individual patients and their families went far to support the genetic theory that sickle cell anemia is a disease of the homozygote. Contradictions to this hypothesis led to the discovery

phoretic patterns of hemoglobin samples for several combinations of these hemoglobins appear in Fig. 34-6

Although Hb C trait is completely harmless the homozygous state for Hb C is definitely a disease. Under favorable conditions the hemoglobinopathy is latent but the decreased life span of the erythrocytes will exacerbate any additional stress. It is of clinical importance that sickle cell-Hb C disease and Hb C disease are associated with splenomegaly whereas in adults with sickle cell anemia the spleen cannot be felt. In sickle cell anemia numerous splenic infarcts eventually lead to autsplenectomy. The cells of Hb C disease are thin and numerous target cells can be seen (Fig. 34-7).

Since the characterization of Hb A, S, and C, numerous other human hemoglobins have been described. Their classification is complex and will be discussed in detail further on under Nomenclature of Human Hemoglobins. It may be helpful at this stage to survey what is known of the molecular changes which underlie the variations in the globin molecule.

#### THE POLYPEPTIDE CHAINS OF THE DIFFERENT HUMAN HEMOGLOBINS

Ingram's finding that the difference between hemoglobins A and S is in the amino acid sequence of the polypeptide chains forming the globin molecule resulted in an entirely new insight into the nature of protein polymorphism. The abnormality in sickle cell hemoglobin might well have been physical and could have been caused by a difference in folding of the polypeptide chains.

Indications of a difference in the free carboxylic residues had come from an ingenious differential titration by means of paper electrophoresis [41-42]. It had been calculated from the difference in isoelectric point that Hb S differs from Hb A by possessing two to four more net positive charges per molecule [17]. Scheinberg [41-42] brought forward evidence that the difference is due to a smaller free carboxylic group content in Hb A. On electrophoresis at pH 11.7 the two pigments differ in their mobility and since lysine and arginine would lose their electrical charge at pH 11.7 the difference cannot be due to one of these amino end groups. On the other hand at pH 4 and below free carboxylic groups would lose their charge and at this pH Hb A and S fail to differ on electrophoresis. From this it could be concluded that Hb S carries a smaller number of aspartic or glutamic acid residue or both. Such a difference of over all charge could have been due to an arrangement by which both globins were chemically identical but differed in the arrangements of the polypeptide chains so that fewer free carboxylic groups could influence the over all charge in hemoglobin S or it could have been due to a difference in chemical nature. Hb S possessing a lesser number of free carboxylic

sequence is that heterozygotes for these two abnormal hemoglobin genes suffer from sickle-cell disease. The anemia is modified because the total amount of Hb S in the cells is still less than that formed in the homozygous sickling condition. Thus the genetic approach had led to the discovery of a

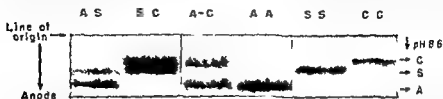


Fig 34-6 The identification of different hemoglobins by filter paper electrophoresis. In an alkaline buffer hemoglobin will travel towards the positive pole. The speed of migration differs: adult hemoglobin (A) is faster than sickle hemoglobin (S) and S is faster than Hb C. Hb D travels at the same speed as S but can be differentiated by its inability to form a gel in the reduced state. (By permission of C. M. Edington et al. [40].)

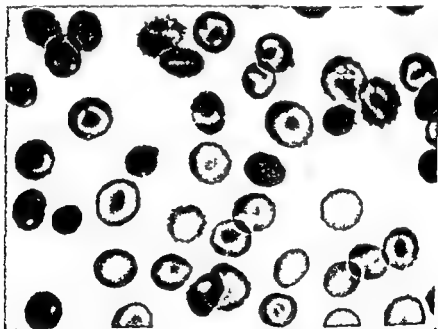


Fig 34-7 The red cells of hemoglobin C disease as they appear in the peripheral blood.

new kind of hemoglobin under genetic control. Hemoglobin C was shown to produce a symptomless trait in heterozygotes, and its proportion in the trait-carrying parents was less than that in the children with Hb S and C. The homozygous state for hemoglobin C was not encountered by Neel, Kaplan, and Zuelzer [37], but they predicted it and it was found shortly afterwards by several other workers. Ranney and her colleagues [39] were able to show that the genes for Hb A, S, and C were allelic. Electro-

acids. Seven of these were identical in both peptides but one—the sixth—was glutamic acid in Hb A and valine in Hb S. This observation confirmed independently and indeed brilliantly the earlier predictions of Pauling [17] and Scheinberg [41–43] that the charge difference between the two proteins could be due to a loss of two carboxylic residues in Hb S, and showed that this variation was of a chemical rather than positional character. The low solubility of Hb S in its reduced state was not explained by Ingram but he pointed out that the solubility of a protein depends upon the distribution of positive and negative charges upon its surface. A change in surface charge pattern through the removal of a glutamic acid could well result in a change of solubility. Ingram's clarification of the chemical difference between the two hemoglobins also indicated for the first time just how a gene might act in the synthesis of polymorphic proteins: for the only alteration caused by the Hb S mutation was the substitution of one in 280 amino acids.

When Hb C was examined by the same methods [50] it was seen that peptide No. 4 was also affected. The Hb A No. 4 peptide had disappeared and two others had taken its place. This indicated that Hb C No. 4 peptide possesses an arginine or lysine in peptide No. 4 where Hb A and S have no bond that can be attacked by trypsin. It was then found that the glutamic acid of Hb A and the valine of Hb S are replaced by lysine in Hb C. Thus a negative charge lost in Hb S is replaced by a positive charge in Hb C. This difference explains the electrophoretic behavior of Hb C which on electrophoresis at alkaline pH (8.6) migrates behind Hb A toward the positive pole about twice as slowly as Hb S. A change from glutamic acid to lysine involves a difference of two units of electrical charge. Furthermore, these findings reinforce the genetic evidence that the two mutations occur in the same place on the gene since they affect the same amino acid. These findings are summarized in Table 34-1.

TABLE 34-1 AMINO ACID SEQUENCE IN PEPTIDE NO. 4  
H<sub>m</sub> globin  $\beta$  chain

|   |     |     |     |     |     |                  |     |     |
|---|-----|-----|-----|-----|-----|------------------|-----|-----|
| A | Val | His | Leu | Thr | Ile | Glu <sup>-</sup> | Glu | Lys |
| S | Val | His | Leu | Thr | Ile | Val              | Glu | Lys |
| C | Val | His | Leu | Tl  | Ile | Lys <sup>+</sup> | Glu | Lys |
| C | Val | His | Leu | Thr | Pro | Glu <sup>-</sup> | Gly | Lys |

Note: Val = valine; His = histidine; Leu = leucine; Tl = threonine; Pro = proline; Glu = glutamic acid; Lys = lysine; Gly = glycine.

An investigation of three samples of hemoglobin D demonstrated the fallacy of identifying a hemoglobin on the basis of its electrophoretic properties alone. This potential source of error had been recognized before and indeed Hb D had been identified as one with the electrophoretic properties of Hb S but without a tendency to cause sickling in the reduced state [11]. Two samples of Hb D which were otherwise indistinguishable—one from a Cypriot and the other from an Indian

groups. Since one molecule consists of approximately 560 amino acids, such a small difference in amino acid composition might well have been thought impossible to establish.

According to the latest estimates human hemoglobin has a molecular weight of 66,700. X-ray studies of horse hemoglobin have shown that the molecule is composed of two equal halves which together form an ellipsoid of the dimensions  $50 \times 55 \times 70 \text{ \AA}$  [43, 44]. In human hemoglobin each molecule contains four polypeptide chains of two types  $\alpha$  and  $\beta$  [45, 46]. The terminal sequence of the  $\alpha$  chains is valyl leucyl, and that of the  $\beta$  chains valyl histidyl leucyl. Since the hemoglobin molecule

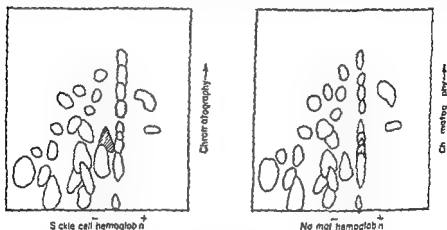


Fig. 34-8. A fingerprint of the polypeptides from Hb A (right) and from Hb S (left). Electrophoresis was performed from left to right and chromatography vertically. Note that the peptides all correspond except for one indicated by the shaded area. (By permission of V. M. Ingram [47].)

consists of two identical halves it would not have been necessary to look for a difference in all the 560 amino acids but only in 260 of them, but that is still a formidable number. Ingram therefore decided to break down the molecule by tryptic digestion and to examine the smaller components separately [47, 48]. Trypsin hydrolysis breaks peptide chains where the amino acids lysine and arginine occur but nowhere else. Since there are approximately 26 molecules of lysine and arginine in each half molecule of globin, the digested protein resolves into 28 short amino acid chains (Fig. 34-8). The peptides were separated in one direction by electrophoresis and in another by chromatography on the same large sheet of paper [49]. These maps or "fingerprints" of the hemoglobin molecule split into small peptide components permitted detection of small differences between specific peptide fragments of Hb A and Hb S. Of the 28 peptide spots all but one were identical in the two fingerprints. Peptide "No. 4" occupied a different position in the fingerprint of the Hb S than of Hb A. Ingram found that this peptide contained eight amino

Hb D and in Hb E and Hb D<sub>s</sub> respectively all four hemoglobins showed individual substitutions characteristic for themselves. Yet a third hemoglobin D (D<sub>3</sub>) differed from both D and D<sub>s</sub> [52] (Fig 34 9).

Ingram now went further and determined in which of the two polypeptide chains  $\alpha$  or  $\beta$  the amino acid sequence was altered. He separated

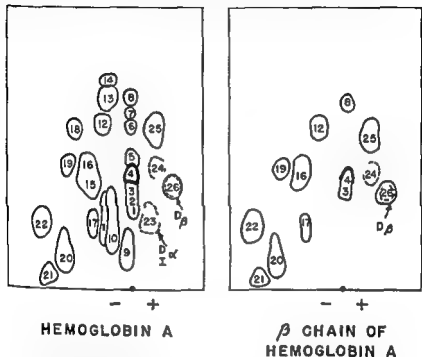


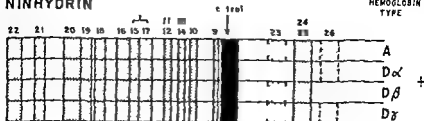
Fig 34-10 Separation of the  $\alpha$  and  $\beta$  polypeptide chains of normal adult hemoglobin (Hb A). On the left is the fingerprint of the globin of Hb A which is composed of  $\alpha$  and  $\beta$  polypeptide chains and on the right the fingerprint of the isolated  $\beta$ -polypeptide chain. Peptides No 4 and 6 which carry the mutational characteristics of Hb S and C and I and D<sub>s</sub> respectively are part of the  $\beta$  chain. Peptide No 3 which is altered in Hb D and I is by inference in the  $\alpha$  chain. (After a sketch by Dr I. M. Ingram.)

the two chains by electrophoresis and submitted them each to fingerprinting (Fig 34 10). His results together with subsequent work with his collaborators [53-55] made it clear that peptide No 4 with the alterations for S and C and peptide No 26 with those for Hb I and D<sub>s</sub> are parts of the  $\beta$  chain and that peptide No 23 which carries the differences found in Hb I and D belongs to the  $\alpha$  chain. Thus the  $\alpha$  chains of hemoglobins A, S, C and I are all identical and normal and the  $\beta$  chains of hemoglobins A, I and D are all identical and normal.

This work found full confirmation from some ingenious hybridiza-

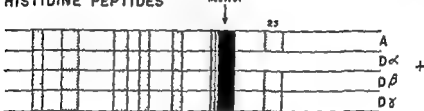


## NINHYDRIN

HEMOGLOBIN  
TYPE

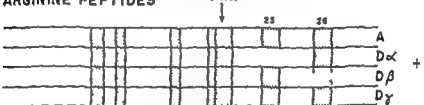
## HISTIDINE PEPTIDES

neutral



## ARGININE PEPTIDES

c 1001



## TYROSINE PEPTIDES

c 1001

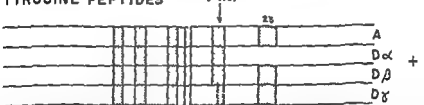


Fig. 34-9 The peptides of three samples of Hb D  $\alpha$   $\beta$  and  $\gamma$ . All three samples were indistinguishable on electrophoresis and chromatography. The fingerprints however were different in all three. The illustration shows schematically the results of staining the fingerprints with ninhydrin and specifically for histidine, arginine and tyrosine residues respectively. (By permission of H. L. Itano [1].)

showed a chemical difference in certain peptides. Some time later when hemoglobins L and I were examined it was found that the change in the first hemoglobin D ( $D_a$ ) was found in the same peptide which was altered in hemoglobin I. In the second ( $D_\beta$ ) there was a difference from Hb A in amino acid composition of the same peptide which is altered in hemoglobin E. Although the same peptide was changed in Hb I and

possess four genes: a normal  $\beta$  chain gene and abnormal  $\alpha$ -chain gene responsible for the Hb P and an abnormal  $\beta$  chain gene and a normal  $\alpha$  chain gene responsible for Hb S. He could then form his three adult hemoglobins A, S, and P as follows:

|   |      |
|---|------|
| Normal $\alpha$ chain gene + normal $\beta$ -chain gene   | Hb A |
| Abnormal $\alpha$ chain gene + normal $\beta$ chain gene  | Hb P |
| Normal $\alpha$ chain gene + abnormal $\beta$ -chain gene | Hb S |

There was in this case no combination of the two abnormal  $\alpha$  and  $\beta$  chains. Indeed experiments designed to produce such a hybrid *in vitro*

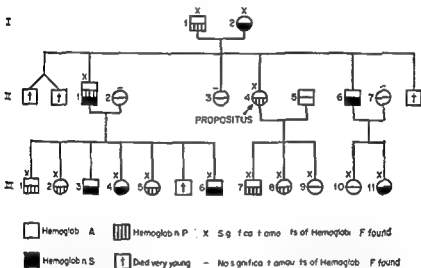


Fig 34 11 The appearance of hemoglobins A, S, and P in one family from the Belgian Congo. The son of a Hb A + S father and an A + S mother had Hb A, S, and P. He married a woman with no abnormal hemoglobins and had by her lot of Hb A + S and Hb A + S offspring. This inheritance pattern supports the theory of two adult hemoglobin genes for man. One determines the  $\alpha$  chain and the other the  $\beta$  chain (By permission of E. W. S. with et al [61]).

were not successful. On the other hand, hybrids could be formed between the non- $\alpha$  chain of Hb I and the  $\alpha$  chain of Hb I using the hemoglobin of another member of this family [63] (Fig 34 12).

With one exception, Ingram's theory has stood the test of what was known before and what has been discovered since. In a case of Hb G, which in this study came from an American family with Italian origins [64] and was probably not identical with the West African Hb G of Fodington and Lehmann [66], the  $\beta$  chain was found to be abnormal [67]. If this is true, it could not be understood on Ingram's theory that the genes for Hb S and his Hb C are nonallelic, as suggested by Neel [60]. A close

tion" experiments carried out respectively by Itano and Schroeder and their collaborators Singer and Itano [56] had observed that human hemoglobin dissociates at pH 4.3 into two unequal halves. One might have expected to find the two symmetric half molecules which had been recognized in x-ray studies each consisting of one  $\alpha$  and one  $\beta$  chain  $\alpha\beta$  and  $\alpha\beta$ . However, at acid pH the two halves which were obtained were asymmetric and consisted of the two  $\alpha$  chains and the two  $\beta$  chains respectively  $\alpha_2$  and  $\beta_2$ . When mixtures of Hb A and Hb S [56, 57] were submitted to this dissociation of the chains and when these were then recombined by changing the pH from acid to alkaline, the subunits of the globin molecule combined indiscriminately. The  $\alpha$  chains from Hb A ( $\alpha_1^A$ ) combined both with the  $\beta$  chains from Hb A ( $\beta_1^A$ ) and from Hb S ( $\beta_1^S$ ) and vice versa. Of the four possible combinations only those with the  $\beta$  chains of Hb S had the properties of sickle-cell hemoglobin regardless of whether the  $\alpha$  chains were derived from Hb A or Hb S. Thus both from Ingram's direct analysis and from the hybridization studies, it may be concluded that the  $\alpha$  chains of Hb A and Hb S are interchangeable. A logical next step was to attempt hybridization of an abnormal  $\alpha$  chain with an abnormal  $\beta$  chain in order to see whether this would result in a new and hitherto unobserved hemoglobin. Itano succeeded in preparing such pigments by dissociating and recombining mixtures of hemoglobins I<sup>S</sup> and C [58] and was even able to combine the asymmetric subunits of human and canine hemoglobin molecules producing thereby two new hemoglobins [59].

Ingram [53] proposed a new genetic interpretation of the inheritance of human hemoglobin on the basis of these findings. The possibility that there might be two hemoglobin genes had been put forward by Neel on the basis of a family study in which it was concluded that the genes for Hb S and G cannot be alleles [60]. Smith and Torbert [61] found in a single individual Hb A and S, and a 'fast' hemoglobin. Similarly Cabannes and Portier [62] found a family where Hb D and H were inherited independently and together with Hb A. Dherte, Lehmann and Vandepitte [63] observed a man who possessed hemoglobins S, P and A and who on marrying a normal wife had children with the traits for both Hb S and Hb P. Another individual with Hb A<sup>S</sup> and P was examined independently, but no family study could be carried out [64] (Fig. 34.11). Ingram suggested that the two hemoglobin genes might correspond to the two peptide chains of human globin, one determining the  $\alpha$  chain and the other the  $\beta$  chain. The alterations in hemoglobins S, C, D<sub>s</sub> and E would then be the outcome of mutations in the  $\beta$  chain gene and those seen in D<sup>I</sup> and presumably P would be caused by mutations in the  $\alpha$  chain gene. One could then readily understand why the son of a marriage of a sickle cell trait carrier with a Hb P trait carrier should possess three hemoglobins (cf. Fig. 34.11). If he inherited Hb P and S he would

fundamental difference between adult hemoglobins and Hb F. Schroeder and Matsuda [70] found that in addition to terminal valyl residues, Hb F contains chains with glycyl residues ( $\gamma$  chain). The valyl chain is in fact the  $\alpha$  chain of normal adult hemoglobin (valyl leucyl) [71]. It can now be understood why there are such profound differences between the adult hemoglobins and Hb F. These differences extend to a shift in the position of the tryptophan fine spectrum band and to altered immunologic properties. Unlike Hb A and S, for example, Hb A and F do not differ by two amino acids per molecule, but rather one-half of each is composed of an entirely different polypeptide complex. Certain differences in products of tryptic digestion of Hb A and F have also been observed by Katz and Chernoff [72]. They observed 19 of 30 possible peptides and found that 11 of these were the same in Hb A and Hb F. An abnormal fetal hemoglobin, first seen at St. Bartholomew's Hospital in London and called hemoglobin Barts [73], was found to be the exact fetal equivalent of Hb H [74]. It consisted entirely of the glycyl chains of hemoglobin F in the form of a tetramer. Hemoglobin H ( $\beta_4$ ) may be found without Hb Barts ( $\gamma_4$ ) and vice versa, but a family has been studied in which the mother and one of her four children had Hb A, H Barts, and F [75]. More Hb Barts was present than Hb H, and whereas Hb A amounted to more than 80 per cent of the total, Hb F was found only in traces. This suggests that if there is a shortage of  $\alpha$  chains, less would be attached to  $\gamma$  chains than to  $\beta$  chains.

On the basis of what is known of the polypeptide chains and their amino acid sequences, one can now classify hemoglobins into those which possess an  $\alpha$  chain and those which do not. Those which do not are Hb H and Barts. Alpha chain hemoglobins can then be subdivided into those with and without a  $\beta$  chain. Those with a  $\beta$  chain are all the hitherto examined adult hemoglobins (except Hb H) and that without a  $\beta$  chain is Hb F ( $\alpha_2\gamma_2$ ). The  $\alpha\beta_2$  hemoglobins can be subdivided into normal adult hemoglobin and two other groups: the first carrying a mutational alteration in the  $\alpha$  chain (D, I, P, Q) and the second with mutational alterations in the  $\beta$  chain (S, C, D $\beta$ , E, G, J, L, and V). Responsible genes would be the following:  $\alpha$  chain gene,  $\beta$  chain gene,  $\gamma$  chain gene, and at least one gene concerned with the amount of  $\alpha$  chains to be produced. This gene or another would also control the allocation of the  $\alpha$  chains to either  $\beta$  or  $\gamma$  chains.

## NOMENCLATURE OF HUMAN HEMOGLOBINS

It is clear that the time will come when hemoglobins will be classified and named according to their polypeptide chains and the amino acid sequences they contain. Meanwhile, a more empirical nomenclature will have to be used. The position is not unlike that of the blood groups.

examination of the experimental evidence for this non allelism [65] suggests that this problem requires a careful study.

On the basis of Ingram's theory it should be comparatively easy to determine whether an abnormal hemoglobin is due to an  $\alpha$  chain or a  $\beta$  chain mutation. A fingerprint would show whether the alteration occurs in a peptide belonging to one of the two chains. Further, hybridization with Hb S or C ( $\beta$  chain mutation) would result in a new abnormal hybrid if the hemoglobin under test is the outcome of an  $\alpha$  chain mutation and hybridization with Hb D<sub>e</sub> or I ( $\alpha$ -chain mutation) similarly.

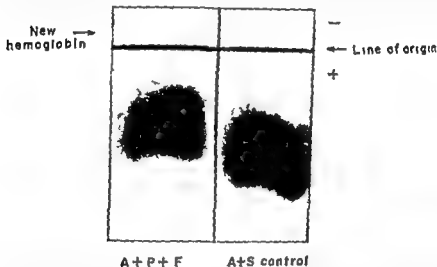


Fig 34-1. A hemoglobin hybrid. The propositus of the family illustrated in Fig 34-11 possesses Hb A ( $\alpha_2\beta_2$ ), Hb I ( $\alpha^F\beta_2$ ), and Hb F ( $\alpha_2\gamma_2$ ). On dissociation of the peptide chains at pH 4.6 and recombination at pH 8.6, a new hemoglobin can be seen on electrophoresis, presumably  $\alpha^F\gamma_2$ . On the right is a hemoglobin A + S control. (By permission of E. W. Smith et al. [61].)

would give a new abnormal hybrid if the test hemoglobin is the result of a  $\beta$ -chain mutation. Such experiments have by now been carried out with Hb I, N, I, O, K, Norfolk, and Q and have indicated that the first four must carry their abnormality of amino acid sequence in the  $\beta$  chain, the others belong to the hemoglobins with abnormal  $\alpha$  chains [155].

Yet another type of abnormality has been established in the case of Hb H. It differs from the other adult hemoglobins in that it is absent from the blood of parents of persons with Hb H, but it is occasionally found in a sibling, or grandparent. It does not seem to appear unless a gene for thalassemia is also present [63]. Jones et al. [69] have recently reported that Hb H consists entirely of normal  $\beta$  chains. Since its molecular weight does not differ from that of Hb A, the  $\beta$  chains must be associated in a tetramer.

Investigation of the polypeptide chains has also thrown light on the

All the hemoglobins (with the possible exception of hemoglobin M) have been discovered because they showed electrophoretic properties different from hemoglobin A. It must be expected that there are many more in which substitutions in the peptide chains do not alter their electric charge and also that there may be hemoglobins with identical electrophoretic properties which do not have the identical amino acid sequence. Indeed this has already been shown for Hb D for when other wise identical samples were submitted to an analysis of their peptides after tryptic digestion [52] sequence differences were observed. Electrophoretic differences are often small and it has been suggested that some



Fig 34-13 Separation by resin chromatography (Amberlite IRC 50 pH 6) of hemoglobins which on electrophoresis at pH 8.6 move similarly or identically. In this instance chromatography was used to identify an unknown hemoglobin C36% as Hb G. The order of electrophoretic mobility at pH 8.6: A Q G L = P D is on chromatography A = P G D Q L. Hb P and Hb A form a broad band. (By permission of J. A. Hunt [14].)

of the electrophoretically abnormal variants might be artifacts [89] but when the variants occur in families the evidence is convincing for a new type of hemoglobin. Electrophoresis at varying pH and chromatography on Amberlite IRC 50 [94] have been useful in differentiating hemoglobins which have closely similar electrophoretic properties at alkaline pH. A chromatographic comparison of several hemoglobins appears in Fig 34-13.

Ager, Lehmann and Vella [89] have proposed the following classification as a temporary solution. They divided the human hemoglobins into two major groups: fetal and adult. The fetal group, which shows an ultraviolet spectrum of the fetal type, includes Hb F, the pigment of Fetus and Papaspyrou [95]  $\gamma_4$  (Barts) and Alexandra. The adult variants are divided into six groups according to their behavior on paper electrophoresis at alkaline pH. These groups can be established by using as markers normal hemoglobin and Hb  $\beta_4$  (H) and S. Erythrocytes containing Hb  $\beta_4$  can be identified independently of electrophoretic methods because cells containing this hemoglobin form inclusion bodies upon

Although much information has been gained about the chemical nature of the agglutinogens A and B, the H substance and the Lewis substance which coats the red cells blood groups are still identified according to their agglutinins

In 1953 Chernoff and his colleagues [76] proposed that for mnemonic reasons the following letters be assigned to hemoglobin known at the time

- F Fetal hemoglobin
- A Normal adult hemoglobin
- S Sick cell hemoglobin

Later Singer [77] proposed that the letter M should be used for the abnormal methemoglobin of Horlein and Weber [16]. All other hemoglobins which might be discovered later were to be known by letters of the alphabet in the order of their discovery beginning with the letter C (b or B had at one time been used for the sickle cell hemoglobin). Difficulties arose when different hemoglobins were given the same letter by their discoverers or when two or more designations were suggested for the same hemoglobin. Thus hemoglobin A<sub>2</sub>' is still being called A<sub>2</sub> by some investigators [14] and B<sub>2</sub> by others [78]. It is doubtful whether Hb G discovered in West Africa [66] is the same as Hb G discovered by Schwartz and his colleagues [65]. Cabannes and Buhr [79] named a 'fast' hemoglobin discovered by them in Algeria Hb I not knowing that this letter had been allotted by Rucknagel and his colleagues [80] to a hemoglobin which they had originally named Hb H [91]. Rucknagel and coworkers had not been aware that the letter H had been given to a newly discovered hemoglobin still known by this letter today [82]. Cabannes et al [83] altered their designation from I to J, but then found that this letter had been given to another newly discovered hemoglobin [84]. Eventually it was decided at the Sixth Congress of the International Society for Hematology to rename this hemoglobin for a third time and it has since been known as Hb K [85]. In 1956 Robinson et al [86] suggested a way out of this dilemma by naming two hemoglobins 'Liberian I' and 'Liberian II' after the place where they had been discovered. Later these were designated Hb N and K [73]. In this way Hb P was first known as the 'Galveston' type [87] and Hb O as 'Buginese' [88]. Hopkins I' and 'Hopkins II' seen at Johns Hopkins Hospital in Baltimore [61] are presumably hemoglobins I and J. Hemoglobin Norfolk [89], hemoglobin Sud Vietnam [90] and Stanleyville I and 'Stanleyville II' [91] are other examples. Two abnormal fetal hemoglobins have been named after the hospitals where they were first seen: Bart's after St Bartholomew's Hospital in London [73] and Alexandra after the hospital of that name in Athens [92]. For Bart's the designation of γ<sub>4</sub> will have to be used in the future [74].

and well known hemoglobins by their present names S C D F etc but the more obscure pigments might well be labeled by giving them a group number or letter followed by a small letter. If after such a scheme was adopted a hemoglobin was found which moved for example between hemoglobins E A<sub>2</sub> on the one hand and hemoglobin O on the other (C group 3a and 3b and C group 4) it would be classified as C group 3<sub>4</sub>. If one wished to place a hemoglobin between the hypothetical pigment C group 3<sub>4</sub>' and O (C group 4) it could be designated C group 3<sub>4</sub>//4.

## VARIANTS OF FETAL HEMOGLOBIN

### PRE-FETAL HEMOGLOBIN

It has been claimed that just as Hb F precedes Hb A in human development so another more primitive hemoglobin precedes Hb F in the fetus. There is no evidence that Hb F precedes the adult pigment in the true sense of the word for although the proportion of Hb I is higher in the embryo than at birth Hb A has been found in the youngest embryos examined.

There is evidence both in favor of and against the existence of a pre-fetal primitive hemoglobin. Allison's primitive hemoglobin P [97] was not a human hemoglobin but was found in rat embryos [98]. In man investigation is difficult because of the small amount of blood which can be obtained from embryos when they become available. Halbrecht and Klubanski [99-100] found in embryos an alkali resistant hemoglobin with a lower mobility on paper electrophoresis at pH 8.6 than that of either Hb F or A. Kunzer and Drescher [101-103] also found a primitive alkali resistant hemoglobin with an ultraviolet spectrum of the Hb I type. Other workers have failed to find either in embryonic or cord blood any hemoglobin other than A and I [104-106].

### HETEROGENEITY OF FETAL HEMOGLOBIN

Vella [107] found in addition to a principal fraction two small components of hemoglobin in cord blood. Since his observations were made by paper electrophoresis it cannot be said at present in which way they are related to minor fractions observed on chromatography [94-108-110]. Indeed chromatographic results are not entirely consistent among themselves. Vella considers the two small fractions the same as those seen by him and others on occasion in greater quantity and described as abnormal fetal hemoglobin. One is the fast moving pigment of Lessas and Lapayrou [91] and the other hemoglobin Alexandra [92]. Thus Hb I would appear to be heterogeneous just as is Hb A where in addition to the main component there are smaller fractions A<sub>2</sub> and A<sub>1</sub>. Traces of Hb Alexandra can be found in all cord bloods but an increased



incubation with cresyl blue. The electrophoretic properties are summarized in Fig. 31.14. Sickle cells can almost readily be identified upon incubation under reducing conditions. Hemoglobin A is identified as that occurring in a number of normal controls. With the help of Hb A $\beta_4$  (H) and S six groups of adult hemoglobins can be established. These

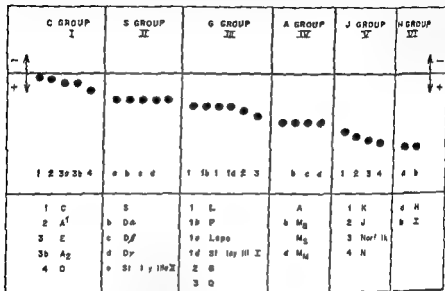


Fig. 31.14. A diagram showing the pattern of adult hemoglobins on paper electrophoresis at pH 8.6. (By permission of A. L. Ogden et al. and the Honorary Editors of the *Proceedings of the Royal Society of Medicine* [98].)

may be numbered I, II, III, etc. or they may be given the name of the first hemoglobin discovered within the group.

|           |                                       |         |
|-----------|---------------------------------------|---------|
| Group I   | Hemoglobins moving more slowly than S | C group |
| Group II  | Hemoglobins moving like S             | S group |
| Group III | Hemoglobins moving between S and A    | G group |
| Group IV  | Hemoglobins moving like A             | A group |
| Group V   | Hemoglobins moving between A and H    | J group |
| Group VI  | Hemoglobins moving like H             | H group |

Within these six groups hemoglobins could again be numbered according to their mobility or when they have the same mobility they could be given (according to the time of their discovery) the additional letters a, b, c, etc.

When such a scheme is accepted a newly discovered hemoglobin could then be allotted to one of the six groups. Clearly a digit should be assigned only after full comparison and international agreement. It is obvious that it would remain the general practice to refer to the common

3 *Hemoglobin Bart's* ( $\gamma_4$ ) On electrophoresis at pH 8.6 this hemoglobin [73-74] moves faster than J and more slowly than H similarly to Hb N. On chromatography at pH 6 it moves fastest of all known hemoglobins. It was thought to be an abnormal fetal hemoglobin because of its absence in both parents because of its resistance to alkali denaturation (intermediate between those of Hb A and Hb F) and because of its ultraviolet spectrum which was of the Hb F type. Hemoglobin Bart's disappears as the child grows older. It has been seen in approximately 50 cord bloods in various parts of the world.

There are now also records of Hb Bart's in an older child and in adults. The first was a Creek boy with thalassemia [112]. The two adults were

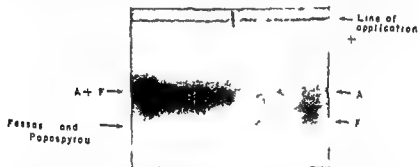


Fig. 34-15. The Papanispyrou abnormal hemoglobin. Paper electrophoresis at pH 8.6. On the left, cord blood; and on the right, Hb A and J. The fast-moving hemoglobin in the cord blood moves similarly to Hb J. Its ultraviolet spectrum is of the Hb F type.

a Jewess from Baghdad and her daughter [75]. In these patients Hb Bart's ( $\gamma_4$ ) was found together with Hb H ( $\beta_4$ ) so that these two women exhibited a dual  $\alpha$  chain deficiency in adult and fetal hemoglobin. One might expect such a dual deficiency to occur more often and there are indications that this might indeed be the case. Huisman [115] found that Hb H resembles Hb F in amino acid composition, particularly in its high isoleucine content (A 0.03 per cent, F 1.85 per cent, and H 1.2 per cent). Since the  $\alpha$  chains of Hb F and Hb A are identical, one may presume that the higher isoleucine content of Hb F is due to its  $\gamma$  chain. Since Hb H is now known to be the tetramer of the  $\beta$  chain [69], Huisman's observation is difficult to understand. His isolation of Hb H was by electrophoresis on a starch slab, a method in which Hb Bart's at the time not yet described might well fuse into Hb H. If Huisman's preparation of Hb H also contained an undetected admixture of Hb Bart's, he would have found a fetal type of amino acid composition in his analysis. A report from Thailand [116] mentions two fast hemoglobin components found in thalassemia hemoglobin H disease and states that the second component resembles Hb Bart's, but the details have not yet appeared.

amount would be considered an abnormality just as an increased Hb A<sub>2</sub> is considered abnormal

### ABNORMAL FETAL HEMOGLOBINS

Fessas and Papaspyrou [91] observed in an infant a fast moving hemoglobin which disappeared as the child grew older. This pigment was described as moving at alkaline pH on paper electrophoresis between A and J. At the same time Vella observed a hemoglobin in cord blood which was sent to Athens and considered by Fessas to be identical with the pigment he had described. This hemoglobin was then referred to as the hemoglobin of Fessas and Papaspyrou in order to differentiate it from another faster moving fetal hemoglobin Hb Bart's. Recently Fessas has observed more examples of a fast hemoglobin in cord blood and although a comparison with his original specimen is no longer possible he now considers that this hemoglobin was in fact identical with Hb Bart's and not with the hemoglobin seen in Singapore by Vella [107]. The designation of a hemoglobin which unlike Hb Bart's moves more slowly than Hb J on electrophoresis at pH 8.6 as "hemoglobin of Fessas and Papaspyrou" may therefore have to be abandoned. The question of renaming Hb Bart's does not need to arise since this has now been identified as the  $\gamma_2$  hemoglobin (i.e. a tetramer of  $\gamma$  chains) and it will probably be referred to as such in the future [74].

Three hemoglobins other than  $\Gamma$  are thus known which exhibit features similar to those of Hb F. The first two may possibly be present in normal cord blood but only in low quantity.

1 *Hemoglobin of Fessas and Papaspyrou* [95-107] This is the least well-defined abnormal fetal hemoglobin. On paper electrophoresis at pH 8.6 it moves faster than Hb A but more slowly than Hb J and on chromatography at pH 6 it moves between Hb  $\Gamma$  and H. The ultraviolet spectrum is of Hb  $\Gamma$  type. Investigators either have not found it to be alkali resistant [95] or have not examined it for alkali resistance [107-112]. Recent observations have been made in Indonesia [113] and in Nigeria [114].

2 *Hemoglobin Alexandra* [92] This hemoglobin moves on paper electrophoresis at alkaline pH more slowly than Hb S and faster than Hb C, i.e. it is similar to the Hb O (Fig. 34.15-34.16). Unlike Hb O it behaves chromatographically on agar electrophoresis at pH 6 like Hb F, and moves faster than Hb A. The ultraviolet spectrum is of the Hb F type. It is more resistant to alkali denaturation than Hb A.

This hemoglobin has been described twice: once in a Greek and once in a Chinese infant. If there should be a connection between the observations of a slow moving prefetal hemoglobin [99] and the so-called abnormal fetal hemoglobins it is most likely that this would relate to Hb Alexandra.

Gamma<sub>4</sub> hemoglobin was originally discovered in an infant of mixed Caucasian Negro Chinese extraction. The second child observed in Britain was also of West Indian origin and the parents showed both Negroid and Mongoloid features [117]. Further instances were seen in a sample of cord blood from Nigeria [114] and in one from a Negro infant from Texas [118]. Two cases of  $\gamma_4$  were observed in Chinese infants in Singapore [107]. In Thailand 22 of 415 cord bloods contained this abnormal fetal component (two were twins) [116]. It was in this study that



Fig 34-17 Two fast moving gel normal fetal hemoglobin gels. On the left is the hemoglobin of Fessas and Papaspyrou and on the right hemoglobin Bart's. Paper electrophoresis at pH 8.6.

evidence for a possible association between the two  $\alpha$  chain deficiencies was presented, but the authors also record that they have seen one infant with only one  $\gamma_4$ , whereas a sister showed only the other  $\beta_4$ .

Most of the cases of  $\beta_4$  have been recorded in Chinese and in Greeks. It is remarkable that the same association occurs both for Hb Alexandra and for  $\gamma_4$ . The only Caucasians with  $\gamma_4$  so far seen have been Greeks. In addition to the Greek boy with thalassemia already mentioned [112] Fessas [119] states that he has seen numerous instances of this hemoglobin in Athens and indeed as stated above considers that his first report [9] of an abnormal fetal hemoglobin was one of  $\gamma_4$  rather than of the other fast abnormal fetal hemoglobin which has hitherto been referred to as the Fessas and Papaspyrou component (Figs 34-17, 34-18).

## ADULT HEMOGLOBINS

### Highly Occurring Variants 1 S C D I

Hemoglobin 1 is the normal adult hemoglobin. Its own peculiar abnormality is thalassemia, which is the outcome of a reduced ability to form Hb A and does not relate to any of the other hemoglobins. It is of course possible that what is now considered to be a single hemoglobin A

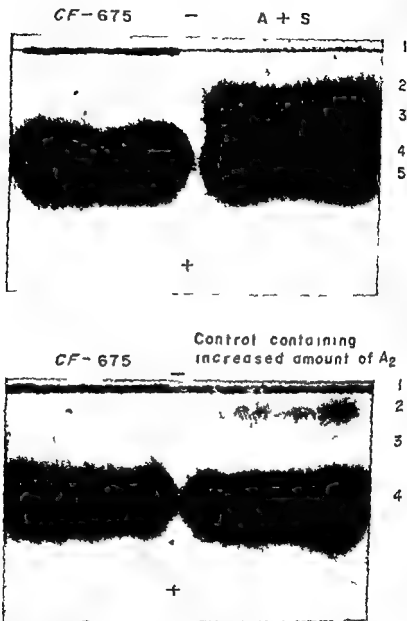


Fig 34-16 Hemoglobin Alexandra moves on electrophoresis at pH 8.6 exactly like hemoglobin O<sub>1c</sub> between S and III (or V). It can be differentiated from hemoglobin O by chromatography at pH 6 and on agar electrophoresis at pH 6.2. By these two methods it shows a greater mobility than V, whereas O moves behind V. In addition Alexandra has a fetal type of ultraviolet spectrum. (By permission of J. A. M. Ager et al [89].)

mia [123] until with the introduction of electrophoretic technique some for example were found to be Hb C disease [123]. Thus whether there is a deficiency of Hb A *per se* as in thalassemia or whether Hb A has been replaced by another hemoglobin the resulting clinical pictures are remarkably alike. An exception of course is sickle-cell disease with its additional complications due to intravascular infarcts. There is other evidence suggesting that a deficiency of Hb A rather than the presence of other hemoglobins causes microcytosis. Whereas trait carriers are asymptomatic double heterozygotes for an abnormal hemoglobin and for

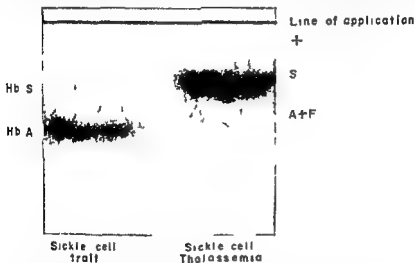


Fig 34 13 Sickle cell trait and sickle-cell thalassemia. The sample on the left shows the sickle-cell trait and that on the right the hemoglobin of a double heterozygote for Hb S and thalassemia respectively. Hb A formation is suppressed when thalassemia is present.

thalassemia (in whom the proportion of Hb A is lower than in simple abnormal hemoglobin trait carriers) resemble clinically homozygotes for the appropriate abnormal hemoglobin. Thus in the case of sickle cell trait they present with sickle-cell thalassemia disease [124-132] and in that of hemoglobin C trait with hemoglobin C thalassemia [133-134] (Fig 34 19). The clinical condition approaches that of the homozygous state more nearly when the relative concentration of the abnormal hemoglobin is higher. This rule is not universal. Hemoglobin F disease tends to be milder than the combination of Hb I trait with thalassemia [135-139]. This is true even though in Hb F thalassemia more commonly than in any other of the doubly heterozygous conditions the suppression of Hb A production is complete. In an Indian family where Hb J and thalassemia were found [140] a double heterozygote showed no suppression of Hb A formation and the proportion of A to J was the

could be a group of different hemoglobins with single amino acid substitutions which do not affect electrophoretic mobility. Ingram et al have in fact suggested that the hemoglobin A in thalassemia might be an abnormal hemoglobin of this type, and that there might be  $\alpha$  chain substituted and  $\beta$  chain substituted hemoglobins A' [170]. This is not impossible because it is known that otherwise indistinguishable samples of hemoglobin D possess different amino acid sequences [59]. The abnormality in thalassemia would be in A<sub>1</sub> the main component. If Ingram's theory of an abnormal hemoglobin indistinguishable at present from Hb A is confirmed then thalassemia minor would be an example of a disability due to an abnormal hemoglobin inherited together with



Fig 34-18 A dual  $\alpha$ -chain deficiency. Comparison by paper electrophoresis at pH 8.6 of a sample containing Hb A and H with one from a woman with Hb A F (trace) Bart's and H. These hemoglobins may alternately be referred to as  $\alpha_1\beta_2$ ,  $\alpha_2$  (trace)  $\gamma$  and  $\beta_4$ . (By permission of I. M. Katz et al [72].)

normal Hb A. With certain qualifications for the sickle cell trait the concept holds at the present time that a trait for an abnormal hemoglobin is harmless.

With one exception the homozygous state for Hb G [121] all homozygotes for one abnormal hemoglobin gene and all double heterozygotes for two different abnormal genes have clinical manifestations of the abnormality. It seems that the absence of Hb A is associated with a flattened cell and a low mean cell hemoglobin content. The abnormality results in a decreased osmotic fragility because the resistance of a red cell to hydration is largely a measure of the difference between the volume of the cell and that of a sphere of equal surface [122]. There is invariably a shortened life span although this investigation has not yet been carried out in all these disorders with equal thoroughness. It may be assumed that this abnormality which is common to all these conditions has a common denominator the absence or shortage of Hb A. It is noteworthy that in thalassemia in which (as it may still be assumed) Hb A is the only adult hemoglobin the pathologic picture is much the same as in the hemoglobinopathies due to abnormal hemoglobins. A number of homozygous conditions were in fact thought to be instances of thalassemia.

tion in the No 4 peptide of the fingerprint the alteration in Hb E occurs in the No 26 peptide (Fig 34 21) The amino acid change explains its electrophoretic mobility at pH 8.6 but not its behavior at pH 6.5. One might conclude that the position of an amino acid substitution in the molecule would influence the electrophoretic behavior as well as the

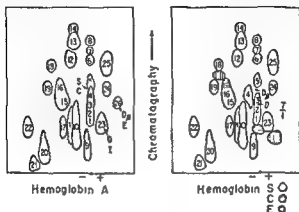


Fig 34 21 A summary of the mutational changes as seen in the peptide fingerprint of Hb S and E (By permission of S H Lee et al [9])

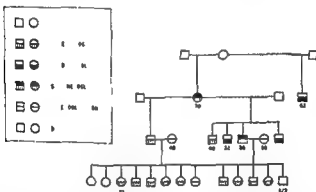


Fig 34 22 The inheritance pattern in a family with Hb A, S, and E. For further locus, see text (By permission of the British Medical Journal [1958])

change in electrical charge. Hemoglobin S and C, which have their amino acid substitution in the same amino acid link of the  $\beta$  chain, are allelic characters [39]. There is only one record of a family in which Hb S and E occurred together [146]. There was conflicting evidence as regards the inheritance of the two hemoglobins (Fig 34 22). The son of a woman with hemoglobins S and E presented the same hemoglobin combination. The father had died. This finding would exclude that the genes for Hb S and E are alleles only if the father had been proved not



same as in persons with nonthalassemic Hb J trait. There was also no evidence of a hemoglobinopathy ("hemoglobin J thal. emia") in this person.

*Hemoglobin D* was first found in a patient with atypical hemolytic anemia. Electrophoresis revealed a single hemoglobin migrating in the position of Hb S [141, 142]. One of the parents failed to show sickle cells on repeated tests, but on electrophoresis a sickle-cell trait pattern was found. It was then discovered that a hemoglobin with the electrophoretic

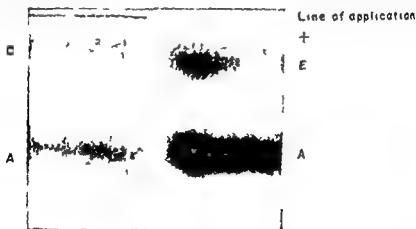


Fig. 34-20. A comparison of Hb C and Hb E tracts. Hb E moves slightly faster than Hb C. Paper electrophoresis at pH 8.6.

properties of Hb S but without its abnormally low solubility in the reduced state was responsible for the conflicting observations. The propositus was in fact suffering not from sickle-cell anemia but from sickle-cell-hemoglobin D disease. The parent was a Hb D trait carrier. At one time thought to be a rare hemoglobin, D is now known to occur in 2 per cent of Punjabis and in 1 per cent of Lohana speaking Gujaratis in southern India [143]. It is now known that "hemoglobin D" is not a single compound but that in this electrophoretically and otherwise identical group several pigments with identical charge but different peptide sequence are included [142].

*Hemoglobin E* was discovered in a family in California [144] and independently in Thailand [135, 145]. The observations in Thailand provided the first evidence that abnormal hemoglobins other than Hb S exist at high frequency in non-Negroids. On electrophoresis at alkaline pH, Hb E is very similar to Hb C, but unlike C it is similar to Hb S on electrophoresis at acid pH (Fig. 34-20). The mutational change in Hb E is in the  $\beta$  chain and as in Hb C involves the substitution of a glutamyl residue of Hb A by a lysyl residue. The peptide affected is, however, not the same in both cases. Whereas for Hb C (and S) there is an altera-

[148] This subject is discussed fully in Chap 33. The variants are named after the localities where they were found  $M_{H(nion)}$ ,  $M_{H(sakatoon)}$ ,  $M_{H(nyctal)$ . Inheritance follows simple Mendelian rules and the affected individuals are thought to be heterozygotes.

*Hemoglobin H* is now known to be  $\beta_4$ . This deficiency of the alpha chain does not become apparent unless there is also a gene for thalassemia [68]. The hemoglobin was discovered in 1933 in a Chinese family [62] and also

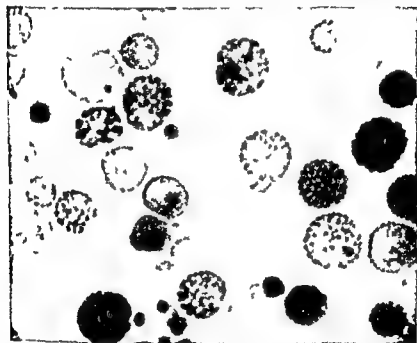


Fig. 34-23. Inclusion bodies in cells containing Hb H. The cells were incubated at 37°C with isotonic cresyl blue. The large inclusions which give the cells almost a nucleated appearance are giant inclusions seen in patients after splenectomy.

in Greece [149]. It has little stability and denatures on storage in the cold. On incubation with cresyl blue, red cells containing this hemoglobin form numerous blue inclusion bodies (Fig. 34-23). These probably represent small foci of denatured hemoglobin.

On paper electrophoresis at pH 8.6, Hb H and I are the fastest moving pigments. At pH 8.0, Hb H is the only known hemoglobin which migrates toward the anode.

*The Lepore hemoglobin* (named after the family in which it was discovered) [150] was found during family studies of cases with thalassemia major. No other examples outside this family have yet been reported. Both parents were of Italian extraction. The propositus, an infant ap-

to carry the sickle cell trait, and about one fifth of the population to which this family belonged are sickle cell trait carriers. On the other hand, in the next generation four children of the man with the two abnormal hemoglobins and of his normal wife had only one abnormal hemoglobin each. Thus on the whole the evidence favors allelism or close linkage of the genes for Hb E and S. Hunt and Ingram [147] state that the locus of the Hb E mutation is probably some distance away from that of the Hb S locus in the gene of hemoglobin. Nevertheless they may be closely linked in the usually accepted genetic sense. The specific chain alterations of Hb A and E are as follows:

Hb A—Val Asp Val Asp Glu Val Gly Gly Glu Ala Leu Gly Arg  
 Hb E—Val Asp Val Asp Glu Val Gly Gly Lys Ala Leu Gly Arg

The known combinations of Hb S, C, D and E appear in Table 34-2.

TABLE 34-2 KNOWN COMBINATIONS OF HEMOGLOBINS S, C, D AND E

| Disease or trait                    | Genotypes | Hemoglobins found |
|-------------------------------------|-----------|-------------------|
| Asymptomatic traits                 |           |                   |
| Sickle-cell trait                   | AS        | A + S             |
| Sickle-cell hemoglobin C trait      | CS        | C + S             |
| Hemoglobin C trait                  | AC        | A + C             |
| Hemoglobin D trait                  | AD        | A + D             |
| Hemoglobin E trait                  | AE        | A + E             |
| Sickle-cell hemoglobin J trait      | JS        | S + A + P + (F)   |
| Sickle-cell diseases                |           |                   |
| Classical sickle-cell anemia        | SS        | E + F             |
| Sickle-cell thalassemia             | AS + thal | S + A + F         |
| Sickle-cell hemoglobin C disease    | SC        | S + C + (F)       |
| Sickle-cell hemoglobin D disease    | SD        | S + D             |
| Sickle-cell hemoglobin E disease    | SE        | S + E             |
| Sickle-cell hemoglobin J disease    | SJ        | S + J             |
| Sickle-cell hemoglobin N disease    | SN        | S + N             |
| Thalassemias                        |           |                   |
| Sickle-cell thalassemia             | AS + thal | S + A + F         |
| Hb C thalassemia                    | AC + thal | C + A + (F)       |
| Hb D thalassemia                    | AD + thal | D + F             |
| Hb E thalassemia                    | AE + thal | E + F             |
| Nonsickling nonthalassemic diseases |           |                   |
| Hemoglobin C disease                | CC        | C + (F)           |
| Hemoglobin D disease                | DD        | D                 |
| Hemoglobin E disease                | EE        | E + (F)           |

Note: (F) = F irregularly found

### *Hemoglobins Associated with a Hemoglobinopathy in the Heterozygote*

Hemoglobin M originally described in 1948 [15] is now known to occur in three variants all distinguished by their spectrum in the visible region

*Hemoglobin Q* was seen in a Chinese family [158]. The first patient also had Hb II. In this patient who suffered from a thalassemia like condition Hb Q replaced the A which is usually seen with Hb II. The only other person observed was the mother of the propositus who showed a hemoglobin mixture of 60 per cent Hb A and 40 per cent Hb Q. The mutational change in Hb Q is in the  $\alpha$  chain. Hb Q moves on electrophoresis at pH 8.6 just faster than Hb G and is again best differentiated from similar slow hemoglobins by chromatography at pH 6 (cf Fig. 34.13).

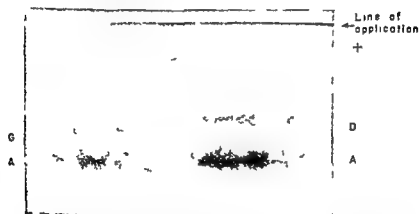


Fig. 34.14 Hemoglobin G on paper electrophoresis at pH 8.6. Hb G moves faster than S and D but separates clearly from Hb A.

*Stanleyville I and II hemoglobins* were seen in a survey in the Belgian Congo [91]. Stanleyville I resembles Hb L and P on electrophoresis at pH 8.6 but moves on chromatography at pH 6 like Hb D. Stanleyville II moves on electrophoresis like Hb D but moves on chromatography behind Hb D. Both hemoglobins were found in the extreme northeast of the Belgian Congo: the first in Nilotes and the second in a Bantu and in a partially Bantu partially Greek family living in a Nilotic area.

### Rare Fast Hemoglobins

*Hemoglobin I* was seen for the first time in an American Negro family where it was present in three generations [91]. It has always been seen in people of at least partial Negro descent. It is probable that a hemoglobin described as Hopkins I [61] is an example of this pigment. This hemoglobin has its mutational alteration in the  $\alpha$  chain in the same finger print peptide as Hb D, peptide No. 23. Hemoglobin I moves on electrophoresis at alkaline pH like Hb II but unlike Hb II moves at pH 6.3 toward the negative electrode.

*Hemoglobin J* was first seen in a young American Negro woman [94] whose father and six siblings also carried the abnormality. Further

parently suffering from thalassemia major was found to possess in addition to Hb A 74 per cent Hb F 12 per cent Hb A<sub>2</sub> and 5 per cent Hb Lepore. Since the mother possessed 11 per cent of Hb Lepore and the father had thalassemia minor with a Hb A<sub>2</sub> concentration of 5.5 per cent, it was concluded that the child suffered from a combination of the two abnormalities. Four other members of the family showed the Lepore hemoglobin in combination with Hb A and a slightly raised level of Hb F (2.3 to 2.6 per cent), they were considered to be heterozygotes.

The presence of some Hb F suggests that the Lepore trait is not simply a genetic replacement of part of Hb A. Gerald and Diamond [150] consider that the Lepore trait is more related to thalassemia than to the abnormal hemoglobin syndromes. It may be that Motulsky's interpretation of Hb H penetrance in association with thalassemia [68] is paralleled by the Hb Lepore trait, and that this pigment is seen in the phenotype only when a gene for thalassemia is also present. It has indeed been suggested that Hb  $\alpha_4$  which would correspond to  $\beta_4$  (H), might turn out to be the Lepore hemoglobin. The only efficient separation from Hb A has been achieved on starch slab electrophoresis at pH 8.6.

#### *The 'Rare Slow Hemoglobins'*

*Hemoglobin G* moves between A and S but separates from A when seen in the trait (Fig. 34-24). This hemoglobin was first seen in a West African family [121]; it may or may not be the same as the Hb G found in a family of Mediterranean extraction [10] which does not separate from Hb A on electrophoresis at pH 8.6. This latter hemoglobin has its mutational alteration in the  $\beta$  chain next to that where the S and C substitutions occur [67]. A hemoglobin with the electrophoretic and chromatographic properties of Hb G has also been seen in a Chinese woman [101]. Hemoglobin G is remarkable for not causing any abnormality in the homozygote.

*Hemoglobin L* moves on electrophoresis at pH 8.0 slightly behind G and in front of S. It was discovered in a family of North West Indian origin [152] and has since been found on seven different occasions always in Indians [103, 154]. Fingerprint studies have shown it to carry its mutational change in the  $\beta$  chain [150].

*Hemoglobin O* which moves on electrophoresis at pH 8.6 between E and S has been seen in 16 of 775 blood samples [26] from Buginese living on the Celebes (Bulawesi) [106]. Family studies have not been possible.

*Hemoglobin P* was first seen in American Negroes [57, 157] and has since been seen repeatedly in the Belgian Congo [63, 64]. It has the same electrophoretic properties at pH 8.0 as Hb I but can be differentiated from it by chromatography at pH 11 (cf. Fig. 34-13). Its mode of inheritance as well as other evidence, suggest that its mutational alteration is situated in the  $\alpha$  chain [63].

*Hemoglobin Q* was seen in a Chinese family [158]. The first patient also had Hb H. In this patient who suffered from a thalassemia like condition Hb Q replaced the A which is usually seen with Hb H. The only other person observed was the mother of the propositus who showed a hemoglobin mixture of 60 per cent Hb A and 40 per cent Hb Q. The mutational change in Hb Q is in the  $\alpha$  chain. Hb Q moves on electrophoresis at pH 8.6 just faster than Hb G and is again best differentiated from similar "slow" hemoglobins by chromatography at pH 6 (cf Fig 34-13).



Fig 34-24 Hemoglobin G on paper electrophoresis at pH 8.6. Hb G moves faster than S and D but separates clearly from Hb A.

*Stanleyville I and II hemoglobins* were seen in a survey in the Belgian Congo [91]. Stanleyville I resembles Hb L and P on electrophoresis at pH 8.6 but moves on chromatography at pH 6 like Hb D. Stanleyville II moves on electrophoresis like Hb D but moves on chromatography behind Hb D. Both hemoglobins were found in the extreme northeast of the Belgian Congo: the first in Nilotes and the second in a Bantu and in a partially Bantu partially Greek family living in a Nilotic area.

### Rare Fast Hemoglobins

*Hemoglobin I* was seen for the first time in an American Negro family where it was present in three generations [81]. It has always been seen in people of at least partial Negro descent. It is probable that a hemoglobin described as Hopkins I [61] is an example of this pigment. This hemoglobin has its mutational alteration in the  $\alpha$  chain in the same finger print peptide as Hb D: peptide No. 23. Hemoglobin I moves on electrophoresis at alkaline pH like Hb H but unlike Hb H moves at pH 6 toward the negative electrode.

*Hemoglobin J* was first seen in a young American Negro woman [84] whose father and six siblings also carried the abnormality. Further

examples have been seen in Indians, Indonesians and West African. Since identification is based solely on electrophoretic properties one might suspect that these widely distant places have given rise to different hemoglobins with the over all charge of hemoglobin J but with different peptide sequences. Hb J moves faster than Hb A and Hb K on electrophoresis at pH 8.6 and does not separate from A at pH 6.5 (Fig. 34.25).

Hemoglobin K moves on electrophoresis at pH 8.6 just in front of Hb A and barely separates from it. It was first seen in Algeria [79] and has also

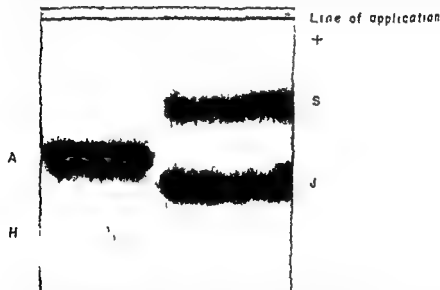


Fig. 34.25. Comparison by paper electrophoresis at pH 8.6 of Hb A + H sample with one containing Hb J and S. Note that the distances S-J and A-H are the same. (The Hb J + S sample was kindly supplied from the first case of this condition seen by Drs J. E. MacIver and L. N. Went, University of the West Indies, Kingston, Jamaica.)

been seen in Liberia where it was described as Liberian II [88]. It has since been seen in other West Africans, in Southern Indians and in Bengalis. It is possible that Hopkins II is Hb K. This hemoglobin was inherited independently from hemoglobin S and its mutational change is in the  $\alpha$  chain.

Hemoglobin N was first seen in Liberia [86] and for a time "Liberian I" was thought to be Hb J. It moves only slightly faster than Hb J on paper electrophoresis at pH 8.6 but differs from Hb J by separating from Hb A at pH 6.5 and also on chromatography at pH 0 [73] (Fig. 34.26). Hb N has since been seen in a few instances in Portuguese territories in the northern parts of West Africa. It is possible that a hemoglobin provisionally described as "R" by Chernoff and found by him in association with Hb S is also Hb N. Hb N has also been seen in association with Hb S in West Africa. The mutational change is in the  $\alpha$  chain.

*Hemoglobin Norfolk* was observed after Hb Q and after the letter R was claimed provisionally for what might in fact have been an instance of Hb N. It was therefore decided to wait with giving the new hemoglobin a letter and it has been named after the county from which the family showing this fast moving hemoglobin was derived. This is an example of an abnormal hemoglobin in an English family with no known foreign connections. Hb Norfolk [89] resembles Hb J on electrophoresis at pH 8.6 and moves only slightly faster though it definitely moves more

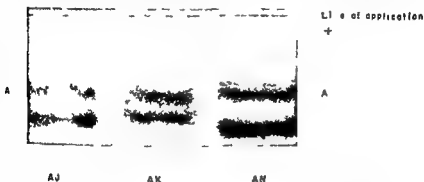


Fig. 34.76. Hemoglobins J, K, and N on paper electrophoresis at pH 8.0. These hemoglobins move faster than Hb A in the order N, J, K.

slowly than Hb N. Hb Norfolk differs from Hb J by separating from A on electrophoresis at pH 6.5. This hemoglobin was discovered in a British airman in Singapore where it was the only abnormal European sample among 550 samples. It was then found in the father and in his brother in Norfolk, England. Its mutational change is in the  $\alpha$  chain. ✓

## WORLD DISTRIBUTION OF ABNORMAL HEMOGLOBINS

Anthropologists are particularly interested in human characters which are not so rare as to be of no general importance nor so widespread that they lose their value in distinguishing among different human populations. For anthropologic purposes perhaps the ideal characters among the abnormal hemoglobins are Hb C and E. Both pigments are present in millions of human beings but at the same time they are peculiarly associated only with certain well-circumscribed groups of people [159]. Hb C discovered in American Negroes could be expected to have come with their ancestors from West Africa but it was surprising to find that Hb C which is not frequent in American Negroes should turn up in 10 per cent of the inhabitants of Accra in Ghana. On the other hand no Hb C is seen in East Africa nor is it found in the Belgian Congo. Occasionally examples have been seen in South Africa or in Central Africa.



but there is always a readily traced connection with West Africa. Within that part of the continent again fairly sharp geographic demarcations are noted. Westwards from Ghana the incidence falls until it becomes very low in Liberia, and there is a similar decline eastwards, where in the Yoruba in western Nigeria the incidence is only about half that found in the coastal region of Ghana. This was even more impressive when it was seen that within Nigeria the River Niger forms a barrier to the spread of

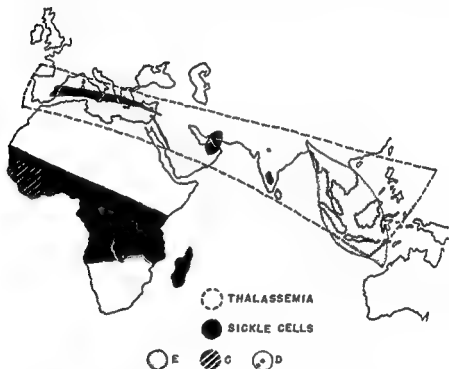


Fig. 34-27 World distribution of major hemoglobin abnormalities

the hemoglobin westwards in the Yoruba. Hb C is found in 5 to 6 per cent of the population east of the river in the Ibo; the incidence amounts to only a fraction of 1 per cent. In Ghana itself the frequency of the Hb C gene increases towards the north; in the northernmost territories of Ghana and in the neighboring French High Volta territory one fifth of the population possess Hb C in their red cells. There is therefore a center of high frequency somewhere in the north of Ghana, with a fall in frequency to the south, east and west. Neel once stated that never in the history of genetics, with the possible exception of Ford's melanism story in the moth, have geneticists and those with kindred interests been quite so close to having a ringside seat at the origin and dissemination of a new gene. (Fig. 34-27)

Hemoglobin E presents a similar situation. Again a very high frequency is found in Burmese and their immediate neighbors but not in Indians in nearby Bengal, Tibetans or Indonesians. There are small foci with a high incidence in the Indonesian archipelago but such a local increase would be expected for any gene in small islands with an inbreeding population. Of particular interest is that Hb F is found in the Indo-Chinese but not in the Chinese and that it is therefore linked with the earlier Mongoloid populations of Southeast Asia. In Malaya there is a higher

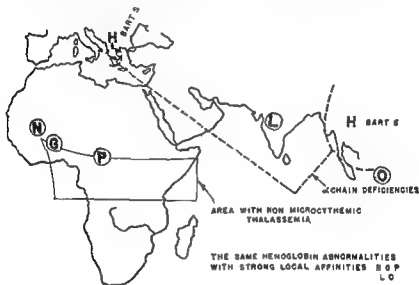


Fig. 34.99. The rare hemoglobin abnormalities with strong local affinities: Hb N, G, L, and O.

incidence in the north. Less hemoglobin E appears in the south where immigration from Indonesia has been of greater importance. Of the primitive populations the Vedda of Ceylon have a high Hb F frequency which links them with the proto-Malays rather than with the Veddoids of southern India in whom sickling but no Hb E is found. This throws an entirely new light on the racial affinities of these Veddoids which is actually borne out by their physical appearance. Of the few proto-Malayan Pygmies examined several were Hb E trait carriers but no Hb E was discovered in the Negritos of the nearby Andaman Islands.

Hemoglobin S is more widely spread. It is found in tropical Africa in the west as well as in the east. In the east there are striking differences between the Hamitic tribes with few if any sickle cells and the Bantus and Nilotes with high sickling rates. There is evidence for a westward spread of the sickling gene and in Liberia sickling is less frequent in the

older and longer-established populations Hb S is also found in India but predominantly in primitive tribes which are the least likely to have been in contact with Africa. It has been proposed that the African continent and India inherited the sickling gene independently but from a common source in the Middle East. This would well agree with what is known of population movements from the Middle East towards the southeast into India.

Hemoglobin D is found at high frequency in India, but it now appears that Hb D<sub>s</sub> is more associated with Europeans in whom it is found occasionally, and that the Hb D itself is not uniform in India either and may be D<sub>s</sub> or D<sub>r</sub>. In India Hb D and Hb L are found in similar populations the first at high and the second at rarer frequency. Although rare whenever Hb D has been discovered in Africa it has been in areas associated with the most westerly regions where it is known to occur. It might be a mutation peculiar to the older inhabitants of that continent.

It is remarkable that the  $\alpha$  chain deficiencies Hb H and Hb Bart's should be seen in Mongoloids such as Chinese and Thais on the one hand and in Greeks on the other (Fig. 34.28).

### POPULATION DYNAMICS OF THE ABNORMAL HEMOGLOBINS

It is tragic that in Africa virtually no homozygotes for the sickling gene reach adult life. Many surveys particularly in East, West and Central Africa have either failed to find sickle-cell homozygotes in the adult population or have discovered them only exceptionally. It is therefore surprising that the gene is present at a high frequency in many parts of that continent [160]. The steady drain caused by the early death of homozygotes would be expected to cause a fall in the incidence of Hb S. Frequencies of 40 per cent and more of the sickle-cell trait can be understood only if there exists a special compensating mechanism which replenishes the Hb S pool in every generation. Neel [161] in 1953 discussed the implications of a situation in which the sickling gene was constantly eliminated by early death of homozygotes but in which a high frequency was nevertheless maintained. One possibility is that a high mutation rate for the sickling gene is a peculiar characteristic of these populations and replaces the genes lost in every generation by sickle-cell anemia. Such an explanation would require a mutation rate about three thousand times greater than those hitherto calculated for man [162] and in a search for evidence to put this theory to the test in tropical Africa no support could be found for it in an extensive survey [163]. The other possibility suggested by Neel is that a balanced polymorphism exists by which the Aa heterozygote enjoys two or more advantages not shared by either homozygote. Allison [162] found evidence that sickle cell

trait carriers enjoy an immunity against malaria which is not shared by the normal homozygote

Several investigators have shared in developing the concept of balanced polymorphism as the genetic mechanism supporting the persistence of the sickling gene at high frequency. Early observations were made in Rhodesia [164-165]. It is of the greatest importance that two points be kept in mind. It is necessary to single out for investigation the effect of *Plasmodium falciparum* and to separate it from the part played by other parasites because only *P. falciparum* causes malignant malaria which kills children in infancy. In these children it is necessary to consider only the age at which immunity against the parasite had not yet been acquired. Immunity is acquired very early in hyperendemic areas and studies of adults or even of children above the age of 2 include many individuals with partial protection against *P. falciparum* some of whom would be sickle-cell trait carriers and normal homozygotes [166]. The protection against malaria afforded to sickling infants could by itself fully explain the high sickling rate in some African populations. The mechanism of protection might be a failure of *P. falciparum* to thrive on sickle-cell hemoglobin or it might be related to the fact that cells infected with this parasite tend to adhere to the vessel wall where they would become deoxygenated. This would cause them to alter their shape and the sickled cells would then be phagocytosed [167]. The latter is the more probable explanation because cells containing Hb S do not differ from normal cells when they are used for culture of *P. falciparum* in vitro [168]. Survival of homozygotes plays little part in maintaining the sickling gene at least in some parts of Africa [169-170]. The protection in infancy of the heterozygotes against malignant malaria is not only the first but also the most striking example for balanced polymorphism acting in the natural selection of the human race.

There is a remarkable correlation between high sickling rate and low social status [171]. The lower the social level of a human group usually the greater is its isolation and inbreeding populations are notoriously likely to accumulate specific characters at a higher frequency than societies with a larger gene pool. There is yet another factor which would tend to raise the sickling frequency in the more primitive tribes in the less accessible parts of Africa. Although the malarial pressure may not be greater the malarial death rate will be increased with unfavorable living conditions [172]. This would explain why the sickling rate is higher in some areas of Africa where the intensity of malarial infection is less than in others with a greater incidence of the sickle-cell trait. With an over all increase in the death rate any differences due to protection against malaria will become more significant. This suggests that the improvement of living conditions as well as the eradication of malaria will cause a fall in the sickling incidence in future generations.

It has not been possible to find a difference in malarial infection between normal homozygotes and carriers of the Hb C trait [173]. It has been suggested that Hb I might be responsible in part for the 'inborn' protection of infants against malaria, as for Hb E and D it has been pointed out that homozygotes who would on the whole be viable and are known to produce children would be protected against thalassemia [174]. Thalassemia acts by interference with the production of Hb A and should therefore have no effect on homozygotes for Hb E or D. In heterozygotes the only conditions which could arise would be Hb I thalassemia and Hb D thalassemia respectively. These hemoglobinopathies are much less severe than thalassemia major. It is noteworthy that both Hb E and D are found in parts of the world where it is now known that the incidence of thalassemia is high and where thalassemia major is the only hemoglobin disease which will cause the death of the affected persons with near certainty before they are able to produce children. Other advantages of Hb E and D might be related to the red cell morphology of the homozygous conditions. The blood picture resembles that of patients with a compensated iron deficiency. There is a polycythemia without a rise in total red cell volume and perhaps such individuals can adapt themselves more easily to an iron deficient diet or to secondary iron deficiency in hookworm disease [143-170].

## THALASSEMIA

Thalassemia is an erythroblastic anemia beginning early in life which in its severe form is associated with enlargement of spleen and liver, a leukemoid blood picture and characteristic bone changes. Throughout the last 70 years the concept of this disease has undergone continuous change. It is now known that the condition described by von Jaksch in 1889 which he called *anemia pseudoleukaemica infantum* and which was latter named *Jaksch Hayem Luzet anemia* included besides thalassemia such different conditions as iron deficiency anemia complicated by bacterial infection and the anemia associated with splenomegaly and cirrhosis (Banti's syndrome).

In 1925 Cooley and Lee [176] described the severe form now known as thalassemia major. The name *thalassemia* or Mediterranean anemia (*θαλασσα* = Mediterranean Sea) was chosen [177] because it seemed at first that the disorder was found exclusively in families of Mediterranean stock. Contribution from Italy and Greece [178-183] prepared the ground for the recognition that there are two similar familial conditions which vary in severity and that the major disability is found in the homozygous state. The milder condition was also described in the United States where it was named *target cell anemia* and *familial microcytic anemia* [184-186].

Here also severity seemed less when the gene responsible for the abnormality was inherited as a single dominant trait [184, 185]

The key investigation which conclusively proved the mode of inheritance was that by Valentine and Neel [187, 188] this study was complemented by that of Silvestroni, Bianco and Villisneri in Italy [189, 190]

### CLINICAL AND GENETIC DEFINITIONS

There are two ways of using the terms *thalassemia major* and *minor*. They are often employed to describe the severe and mild forms of the disease respectively. The term *thalassemia intermedia* has been introduced to describe disease states which are midway in clinical severity between *thalassemia major* and *minor* and *thalassemia minima* has been used when the signs and symptoms are less than in *thalassemia minor*.

When it was thought that all homozygotes suffer from severe *thalassemia* and all heterozygotes from mild *thalassemia* the clinical and genetic interpretations of these terms were taken to be interchangeable (Fig 34-29). Although this is permissible for most cases of *thalassemia* there are a significant number of heterozygotes who suffer from a hemoglobinopathy which can hardly be considered a minor type. Some of these have later been found to combine the heterozygous state for *thalassemia* with that for an abnormal hemoglobin but others have also been proved to be solely heterozygous for *thalassemia*. The explanation is that the *thalassemia* gene is in a sense always dominant and almost always causes some symptoms although the penetrance of the gene may vary. Also since symptoms are present already, however mild they may be they may be exacerbated when additional stress is experienced. A heterozygote for the hemoglobin C gene for example suffers from no known disadvantage. In the presence of bronchopneumonia or of pregnancy the new burden imposed on the hematopoietic system will affect the patient no differently than it would a normal individual. In a *thalassemia* heterozygote however slight symptoms would become exaggerated because the auxiliary mechanisms of blood production have already been called into action and may now no longer be capable of the expansion required for the maintenance of a normal hemoglobin level. Numerous instances of *thalassemia* in heterozygotes have in fact come to notice because of chronic hemorrhage or pregnancy. It would seem more helpful to reserve the terms *major* and *minor* for the genetic types and to describe the clinical conditions as severe, mild or symptomatic. This convention will be employed here.

### PATHOGENESIS

In *thalassemia* the principal disability is failure to produce red cells with normal hemoglobin content. The hemoglobin concentration may not

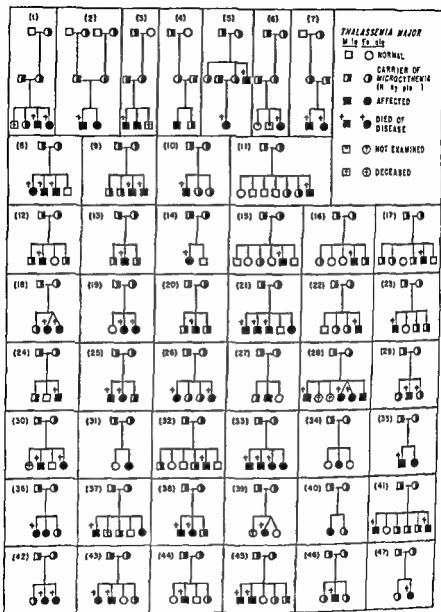


Fig. 31-29. Family trees of patients suffering from thalassemia major (By permission of M. H. Strauss et al. [186].)

be much below normal in these cells but they are reduced in volume. The association of a low mean corpuscular volume with a normal or near normal mean corpuscular hemoglobin concentration is sometimes useful in differentiating the hypochromic anemia of thalassemia from that of iron deficiency. There is a deficiency in Hb A production [191] because of

a disturbance in formation of ferroporphyrin [192 193] or possibly in some instances because of an abnormality in the earlier stages of porphyrin synthesis [104 105]

There is also in thalassemia increased hemolysis in the peripheral blood [196 197] By using  $\text{Fe}^{59}$  to determine the sites of red cell production and to measure bone marrow activity and  $\text{Cr}^{51}$  to measure red cell survival it has been demonstrated that red cell production per unit bone marrow



Fig 34 30 Appearance of the bones in thalassemia major The hypertrophy of the bone marrow causes replacement of the cortex The thinning of the cortex is compensated by numerous small trabeculae giving a mosaic pattern The metacarpals are quadrangular (By permission of Drs A W Franklin and A R Kamp Ho per St Ba tholomew a Hospital London)

is decreased but that total red cell production is greater than normal [198] Destruction of red cells is accelerated and depending upon the equilibrium which the patient establishes between increased production and increased destruction the anemia may be more or less severe At whatever level the equilibrium is finally placed it is obvious that additional blood destruction as in infectious diseases or additional demands as in pregnancy will tend to disturb the delicately balanced state

The clinical manifestations of thalassemia can all be related to the decrease in bone marrow activity per unit active tissue and to the increase in peripheral hemolysis In childhood the first observation in thalassemia is that the patient is pale and listless Intercurrent infections are poorly



resisted Splenomegaly and with lesser regularity, hepatomegaly are noted early. The extension of bone marrow activity into areas of the skeleton which usually do not contain blood forming tissue and the hypertrophy of the bone marrow in those bones where hematopoiesis normally occurs affect the appearance of the skeleton. There is thinning of the cortex of the long bones and the structure is supported by numerous trabeculae (Fig 34-30). X ray study discloses the mosaic pattern of this trabeculation. The diploe of the skull thickens because of marrow hypertrophy, and the outer and inner tables are thin. Perpendicular trabeculation causes the x ray to have a 'hair-on-end' appearance. The thickening of the cranial bones together with a relatively sunken nose, result in a 'mongoloid' facies.

#### APPEARANCE OF THE RED CELLS

None of the features of thalassemia is absolutely constant. Of the various abnormalities changes in the red cells are found with most

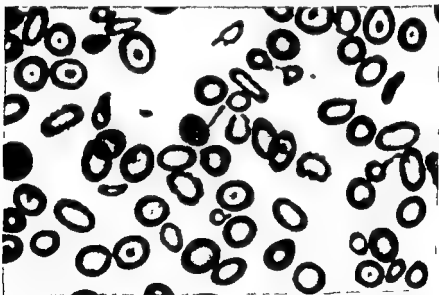


Fig 34-31 Red cells in thalassemia major. Note the thinness of the cells, their unequal size, oval and irregularly shaped cells, and target cells.

regularity. The overactivity of the bone marrow is reflected in the appearance of nucleated erythrocytes in the peripheral blood and in an increase in reticulocytes. The red cells are thin and vary in size. Abnormally shaped cells appear. Since the cells are flatter than normal they tend to appear as 'target cells' in the blood smear and can be recognized *in vitro* by their increased resistance to lysis by hypotonic salt solutions. If the morphologic features of the red cells are used as the criterion for

diagnosis the reduced osmotic fragility is the most useful feature [199] The stimulation of the bone marrow often also results in an increase in white cells to give the leukemoid reaction first noted by von Jaksch (Fig. 34 31)

### HEMOGLOBIN IN THALASSEMIA

Hemoglobin F is nearly always found in thalassemia major and frequently in thalassemia minor It has been doubted whether the alkali-resistant hemoglobin in thalassemia is in fact the same pigment as that found in the newborn but the majority of investigators are now agreed that there are no chemical differences between the two alkali-resistant hemoglobins Further rabbit serum prepared to agglutinate specifically the red cells of the newborn agglutinates also cells of patients with thalassemia [200] Hb A<sub>2</sub> is increased in thalassemia from the usual 2 to 3 per cent to 3 to 5 per cent It can be assumed that both Hb A<sub>1</sub> and Hb F are produced in greater quantity because the quantity of Hb A<sub>1</sub> is reduced A relation has been found between the concentration of Hb A<sub>2</sub> and individual thalassemic families [201] The amount of Hb F however varies widely and members of the same family may show widely different concentrations of this pigment Neither the level of Hb A<sub>2</sub> nor that of Hb F can be correlated in individual patients with the severity of their condition In double heterozygotes for the thalassemia gene and for a gene determining a variant of normal adult hemoglobin such as Hb S or Hb C, only the formation of Hb A seems affected Whereas in a sickle cell trait (AS) more Hb A is present than Hb S the proportion is reversed in sickle cell thalassemia

### HETEROGENEITY OF THALASSEMIA

None of the characteristics of thalassemia can be expected regularly in all cases Gouttas [202] found among 217 heterozygotes that only 99 showed the morphologic features of thalassemia as well as an increase in the Hb A<sub>2</sub> component and a persistence of fetal hemoglobin Of the remainder 109 had the typical red cell picture and an increase in Hb A<sub>2</sub> but with no Hb F Two possessed both abnormalities in hemoglobin composition but had normal red cells and two had abnormal red cells and Hb F but failed to show an increase in Hb A<sub>2</sub> Two showed an increase in Hb A<sub>2</sub> as the only feature and three abnormal red cells without any hemoglobin abnormality A study of parents of patients with thalassemia major showed that the thalassemia gene may be transmitted without any signs of thalassemia [203]

There is now ample evidence that the red cell morphologic changes of thalassemia and the suppression of Hb A production can occur independently The dissociation was first observed in sickle cell heterozygotes Whereas sickle-cell thalassemia usually combines features of sickle cell

anemia with the red cell morphologic appearance of thalassemia, a number of families have now been studied in which the combination of Hb S with Hb I and little or even no Hb A was not associated with leptocytosis.

Parents without sickle cells and children of these individuals may show an increased proportion of Hb I without microcytosis and in some cases without an increase in Hb A<sub>2</sub> [204-208]. Microcytosis without Hb I has been described as a new type of hemoglobinopathy [210] or as 'anemia hypochromica sideroblastica hereditaria' [209]. The finding of Hb I without microcytosis has been termed variously 'nonmicrocythemic thalassemia', 'hereditary persistence of fetal hemoglobin' or the 'I gene' condition. Gerald [148] has gone so far as to postulate a "new" hemoglobinopathy for any thalassemic condition found without an increase in Hb A<sub>2</sub>.

There is no doubt that the dissociation of the features of thalassemia is most easily explained on the basis of a multiplicity of genes which are usually inherited together but which can also be transmitted independently. The inheritance of one 'nonmicrocythemic' and one 'classical' thalassemia gene results in typical thalassemia major [211] as does that of the I<sup>epore</sup> trait together with a single gene for classical thalassemia [201]. Hemoglobin H disease is now recognized as being allied to thalassemia and as requiring the inheritance of at least one thalassemia gene. Yet in this condition Hb A<sub>2</sub> is reduced rather than increased. On the other hand, no noticeable deficiency in Hb A<sub>2</sub> has been noted in thalassemic children with Hb Bart's. This is surprising because both Hb H and Hb Bart's are  $\alpha$  chain deficiencies, one presenting as an excess of the  $\beta$  chain of adult hemoglobin, and the other as a tetramer of the fetal  $\gamma$  chain. In two patients with  $\beta$  chain deficiency in both fetal and adult hemoglobin resulting in both Hb H and Bart's, Hb A<sub>2</sub> was reduced as in Hb H disease.

The multiplicity of the thalassemia genes is borne out further by family studies which have demonstrated that in some families the thalassemia gene is inherited independently of the sickling gene and in others in a manner which suggests either alikism or close linkage [212, 213]. There can be little doubt but that the early suggestions that there might exist several thalassemia genes [8, 214-215] heralded a picture of much complexity. The diagnosis of thalassemia is today a collective concept no less than was the von Jaksch-Hayem-Luzet syndrome two or three generations ago.

#### GEOGRAPHIC DISTRIBUTION OF THALASSEMIA

Most of the early work on thalassemia was on families of Mediterranean origin. There is now no doubt that the term is a misnomer and that thalassemia is most commonly found in Asia. Cases observed in Turks, Persians, Kurdistan Jews [216-218] and even those seen in India [220]

might be related to cases among the Mediterranean populations but this explanation fails to account for thalassemia in Thailand [221]. Although no survey has yet been made in China, Chernoff [222] lists eight accounts of thalassemia in Chinese, most all of whom originated from the area around Canton and Hongkong. One of the authors of this chapter (H. L.) has seen thalassemia in eight Chinese families in London, England. Numerous observations have been made in Indonesia [223] and the Philippines [224].

### POPULATION DYNAMICS OF THALASSEMIA

The homozygote for thalassemia suffers from a severe disease and only rarely survives into adult life. Those who do are sexually immature. Yet in Italy alone the number of thalassemic individuals has been estimated to surpass 1 000 000. A world incidence of many tens of millions would not be an exaggerated estimate.

The high frequency of a gene which for all practical purposes cannot be transmitted by the homozygote and which may cause disease even in the heterozygote requires an explanation. It has been suggested that parents of children who died of thalassemia major might tend to overcompensate their loss. Since both parents would be heterozygous for thalassemia, a greater fecundity from such unions would result in an increase in the number of heterozygotes in the following generation. The theory of overcompensation has been carefully tested in Italy but the available evidence failed to support it [225].

Haldane [175] has made an alternative suggestion that a balanced polymorphism might favor the survival of the thalassemia heterozygote. He suggested that while the abnormal homozygote dies from the anemia, the normal homozygote might be less capable than the heterozygote of adjusting his blood formation to an iron or vitamin deficiency. How such a protection against iron deficiency in the heterozygote might come about has been described by Sjpestejn [226]. Women with thalassemia minor tend to have a mild hypochromic anemia which causes them to increase iron absorption. Since in thalassemia iron utilization for hemoglobin production is decreased, these women would store their iron and eventually would have larger deposits of this element than normal women. They would then be better protected against the iron deficiency anemia of multiparous women which so often is the cause of death in this group in primitive populations. They would also presumably have an advantage when ancylostomiasis causes chronic hemorrhage and subsequent iron deficiency. Haldane also suggested that thalassemia heterozygotes might be better protected against malaria than normal homozygotes and some evidence for this theory has been found in Sardinia [227]. Of two racially identical populations one lived in a highly malarious region and also showed a high frequency of the thalassemia gene and

the other lived in a healthier area and showed a much lower incidence of this abnormality

Whatever the genetic advantage of the thalassemia gene has been in the past or is today in underdeveloped countries the inheritance of thalassemia is now a major problem in some advanced countries where infantile mortality due to other causes such as infection and malnutrition has fallen. Marriage advice has been widely advocated in Italy. The results of the work on bone marrow transfusion and transplantation undertaken in conjunction with leukemia and radiation damage will have to be followed with the greatest attention by those interested in the thalassemia problem and indeed that of the hemoglobinopathies as a whole. One might plan such transplantations for the newborn when antibody formation is still undeveloped. Perhaps the day will come when every child of two parents with the thalassemia trait (or the sickle-cell trait) will receive a bone marrow transplantation from either or both parents at birth without any delay for the diagnosis of the infant's own hemoglobin genotype.

## SUMMARY

1 The hemoglobins of man are a group of pigments each of which is composed of four molecules of ferroporphyrin IX attached to one molecule of globin. The hemoglobins differ in the properties of the globin moiety.

2 At birth fetal hemoglobin Hb F predominates. In normal man this is rapidly replaced by the normal adult pigment Hb A. Hb A consists of at least two components: a major one, A<sub>1</sub>, and a minor component Hb A<sub>2</sub>.

3 The globin molecule consists of two identical half molecules each of which contains two different long peptide chains. In Hb A these are the  $\alpha$  and  $\beta$  chains, and in Hb F the  $\alpha$  and the  $\gamma$  chains. The variant hemoglobins contain either altered combinations of the four chains or altered amino acid sequence within the chains. Each type of chain is thought to correspond to one gene and the inheritance of the polypeptide chains is codominant.

4 Abnormal hemoglobins are principally identified by their electrophoretic properties. The most important abnormal hemoglobin is sickle-cell hemoglobin Hb S, which is responsible for the sickling phenomenon in sickle cell disease. Reduced solubility of Hb S under reduced oxygen tension is responsible for the various clinical and laboratory phenomena of the disease. Heterozygotes for Hb S and Hb A are not anemic, but heterozygotes for Hb S and for other abnormal hemoglobins or for thalassemia may be anemic if Hb S becomes the predominant hemoglobin.

5 Almost all hemoglobins contain  $\alpha$  chains. The exceptions are Hb H, which has four  $\beta$  chains, and Hb Bart's, which has four  $\gamma$  chains. Of the

$\alpha$  chain hemoglobins only one Hb F which is  $\alpha_2\gamma_2$  contains no  $\beta$  chains. The other  $\alpha$  chain hemoglobins are normal hemoglobin tho with an  $\alpha$  chain mutational change (Hb D, I, P and Q) and those with a  $\beta$  chain mutational change (Hb S, C, D, E, F, G, J, L and N). The properties and clinical significance of these and other less well characterized hemoglobins are described in the text.

6 The world distribution of the variant hemoglobins has provided important anthropologic data and has contributed to the study of population dynamics. In the case of Hb S the high frequency of trait carriers is thought to derive from a selective advantage of Hb AS heterozygotes in areas with high mortality from *P. falciparum*.

7 Thalassemia or familial microcytic anemia is not known at present to be the result of an abnormality of hemoglobin structure but rather may be an abnormality in the rate of synthesis of Hb A. The inheritance pattern in the majority of families studied is codominant with and segregating independently of abnormal hemoglobin traits. Thalassemia and abnormal hemoglobins may coexist and their combination is often manifested by anemia.

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## Chapter 35

### The Blood clotting Factors

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*Rosemary Biggs and Lamont W. Gaston*

This chapter is concerned with the clotting of the blood with the specific factors which normally participate in that complicated process and with those diseases which appear to have a genetic basis and which arise because of specific deficiencies in the clotting factors. It must be said at once that the clotting factors are not known as specific chemical species (excepting  $\text{Ca}^{++}$ ) and the reactions which they undergo or catalyze are known only phenomenologically. Accordingly the diseases of the clotting of the blood cannot be given the precise biochemical definition that is the goal of science; that level of understanding must await the painstaking work of the coming years. The reviewers are perforce satisfied to provide a summary of the current status of a problem which is decidedly unfinished business.

Flow of blood from a minor injury usually stops spontaneously and the shed blood clots at about the same time as the blood flow ceases. Moreover, if the blood flow fails to stop in many instances a solid clot also fails to form. These commonplace observations suggest that the clot stops the flow. Indeed the whole superstructure of blood coagulation theory is based upon the assumption that there is a causal relationship between hemostasis and coagulation. It may come as a surprise that this fundamental tenet has been verified only experimentally through the study of hemorrhagic states. This indirect approach to normal coagulation and hemostasis through study of the hemorrhagic diseases is made necessary by unusual experimental limitations. The extent of the limitations must be understood if the problem of hemostasis is to be viewed in reasonable perspective.

Normal blood in a glass tube will clot. Formation of the clot may be observed at different temperatures in recalcified plasma in containers with different surfaces and in plasma with and without platelets. If the sample of blood has defective coagulation the scope for study is immedi-



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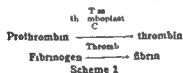
TABLE 35-1 COAGULATION FACTORS AND SYNONYMS

| Most common name                       | Synonym is  | Numerical term |
|--|---|----------------|
| Fibrinogen                             |   | Factor I       |
| Prothrombin                            |   | Factor II      |
| Thromboplastin                         | Thrombokinase   | Factor III     |
| Calcium ion                            | Ca <sup>++</sup>  | Factor IV      |
| Factor V                               | Labile factor proaccelerin aegloulin  | Factor V       |
| Factor VI                              | Term no longer used   | Factor VI      |
| Factor VII                             | Stable factor proconvertin SICV<br>(serum prothrombin conversion accelerator) | Factor VII     |
| Antihemophilic globulin                | AHG antihemophilic factor (AHF)<br>thromboplastinogen platelet cofactor I     | Factor VIII    |
| Christmas factor                       | Plasma thromboplastin component (ITC)   | Factor IX      |
| Stuart factor                          | Lower factor Stuart factor  | Factor X       |
| Hageman factor                         |   |                |
| Plasma thromboplastin antecedent (PTA) |   |                |
| Thrombin                               |   |                |

## THEORY OF BLOOD COAGULATION

## CLASSICAL THEORY

The modern era of blood coagulation research began with the classical theory at the end of the last century. According to the postulated scheme blood clotting follows two simple reactions:



The basis for this formulation was sound and much subsequent work supported it. It is not proposed to discuss its foundation but to take it as an established starting point for modern developments. The history of the theory of blood coagulation has recently been reviewed elsewhere [1].

According to this theory prothrombin is the one precursor of the coagulant thrombin. The reaction is accelerated by a tissue extract called thromboplastin and requires the presence of Ca<sup>++</sup>. The thromboplastin used experimentally is usually a saline extract of fresh or dried brain or lung tissue or on occasion the venom of the Russell's viper (RVV). The venom is a powerful coagulant which differs in its activity

ately increased. Abnormal and normal samples may be mixed. The normal sample may be fractionated to see which component will correct the abnormality in the abnormal sample. When the fraction has been isolated and identified a reconstructed normal system can be devised in which the effects of omitting and including the pertinent fraction can be studied. It is then usually possible to suggest a theory of normal coagulation to include the plasma fractions under study. Invention at this stage may suggest a method for studying and even measuring the abnormality in other similar patients. In this way most of the normal coagulation factors have been discovered and most useful techniques devised.

As more and more different types of defect are studied the number of possible mixture experiments increases and the theory becomes more complex to accommodate all the newly postulated factors in a new normal scheme. This evolution proceeds through phases of comparative clarity when most observed facts fit some hypothetical scheme and phases of revolutionary change when the hypotheses receive complete revision and the whole significance and interpretation of techniques must be reappraised. Thus a test which was used with confidence as a measure of prothrombin at one phase may be interpreted as a quantitative measure of factor V or VII at a later time.

The uncertainty of the whole approach has naturally not commended the subject to the biochemist and it must be admitted that the whole superstructure of theory and hypothetically interpreted methods has become very large. In fact the inclusion of this subject in a book which deals mainly with the biochemical basis of inherited diseases may be unwarranted. However the subject appears to be in an interesting phase which may be approaching transition, a phase when a review emphasizing difficulties and inconsistencies may help to turn the kaleidoscope to a new and more intriguing pattern.

It is proposed to begin the survey with a brief and undocumented account of the history of the theory of blood coagulation and to use it as a skeleton for reference during a more detailed consideration of the coagulation factors and hemorrhagic states. Details of technique will be introduced as the need arises. Finally an attempt will be made to relate the information reviewed to the role of coagulation in hemostasis. A listing of coagulation factors and synonyms appears in Table 35 I.

The subject matter will be limited to those inherited defects which are reasonably clearly defined. For this reason reference to inhibitors of blood coagulation has been omitted; patients with inherited abnormality of the inhibitors of coagulation are too rarely encountered to merit discussion. The platelet defects classed as thrombopathy are omitted because the literature on this subject is still confusing and the different entities are not clearly defined.

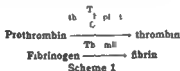
TABLE 35-1 COAGULATION FACTORS AND SYNONYMS

| <i>Not common name</i>                    | <i>Synonyms</i>  | <i>Numerical term</i> |
|---|--|-----------------------|
| Fibrinogen                                |  | Factor I              |
| Prothrombin                               |  | Factor II             |
| Thromboplastin                            | Thrombokinasase  | Factor III            |
| Calcium ion                               | Ca <sup>++</sup>   | Factor IV             |
| Factor V                                  | Labile factor proaccelerin aeglobulin  | Factor V              |
| Factor VI                                 | Term no longer used  | Factor VI             |
| Factor VII                                | Stable factor proconvertin SICa<br>(serum prothrombin conversion<br>accelerator) | Factor VII            |
| Antihemophilic globulin                   | AHG antihemophilic factor (AHF)<br>thromboplastinogen platelet cofactor<br>I     | Factor VIII           |
| Christmas factor                          | Plasma thromboplastin component<br>(PTC)   | Factor IX             |
| Stuart factor                             | Proser factor Stuart Lower factor  | Factor X              |
| Hageman factor                            |  |                       |
| Plasma thromboplastin<br>antecedent (PTA) |  |                       |
| Thrombin                                  |  |                       |

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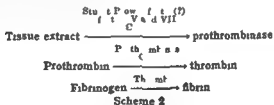
According to this theory prothrombin is the one precursor of the coagulant thrombin. The reaction is accelerated by a tissue extract called thromboplastin and requires the presence of Ca<sup>++</sup>. The thromboplastin used experimentally is usually a saline extract of fresh or dried brain or lung tissue or on occasion the venom of the Russell's viper (RVV). The venom is a powerful coagulant which differs in its activity

from mammalian tissue extracts (see below) Thrombin converts fibrinogen to fibrin which forms the matrix of the clot

#### FIRST MODIFICATION FACTORS V AND VII AND THE STUART PROWER FACTOR

This convenient way of accounting for clotting was jolted by observations made over a period of several years that certain patients with faulty clot formation were defective in one of three specific factors necessary to the rapid conversion of prothrombin to thrombin The *c* factors are now known as factors V and VII and the Stuart Prower factor In these cases examination of the blood showed a slow conversion of prothrombin to thrombin in the presence of tissue thromboplastic extracts and  $\text{Ca}^{++}$  Biggs, Douglas and Macfarlane [2] and Hardisty [3] have shown that factors V and VII in the presence of  $\text{Ca}^{++}$  react with mammalian tissue extract thromboplastin to form an active substance which converts prothrombin to thrombin Owren, Rapaport Hjort and Aas [4] found that the product of this reaction can be sedimented by centrifuging as an *active particulate material*

Stuart Prower factor resembles factor VII in its physicochemical characteristics its deficiency on a hereditary basis gives rise to a hemorrhagic state indistinguishable clinically from factor VII deficiency The mechanism by which these three factors allow tissue thromboplastic extracts to effect the rapid conversion of prothrombin to thrombin is unclear Thus the classical theory had to be rewritten with the product of interaction of tissue extract  $\text{Ca}^{++}$  factors V, VII and Stuart Prower factor being termed *prothrombinase*



The term prothrombinase has been used by Owren [4] for the direct activator of prothrombin Russell's viper venom is not entirely interchangeable with tissue extract in forming prothrombinase The plasma from patients with factor VII deficiency (see below) reacts normally if PVV, lipid<sup>1</sup> and  $\text{Ca}^{++}$  are used brain extract fails to activate the clotting system fully It seems probable that RVV requires lipid and both factor V and the Stuart Prower factor for optimum activity Experiments with

<sup>1</sup> Lipid is any one of several fatty substances such as cephalin

RVV probably have no physiologic significance but it can be employed for diagnostic purposes with various pathologic samples (Table 35 2)

TABLE 35-2 PROPERTIES OF FACTORS I AND VII AND THE STUART PROWER FACTOR

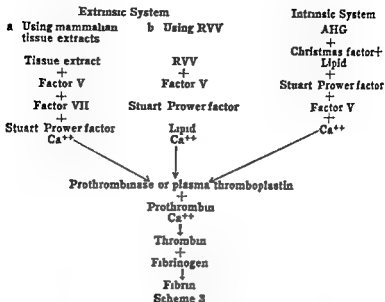
| Properties                    | Factor I     | Factor VII          | Stuart Prower factor |
|-------------------------------|--------------|---------------------|----------------------|
| Stability on storage          | Unstable     | Stable              | Stable               |
| Presence in normal serum      | Absent       | Present             | Present              |
| Adsorption by inorganic salts | Not adsorbed | Adsorbed            | Adsorbed             |
| Required for normal action of |              |                     |                      |
| 1 Brain extract               | Required     | Required            | Required             |
| RVV                           | Required     | <u>Not required</u> | Required             |
| 3 Plasma thromboplastin       | Required     | <u>Not required</u> | Required             |

#### SECOND MODIFICATION HEMOPHILIA AND CHRISTMAS DISEASE FACTORS AND BLOOD THROMBOPLASTIN FORMATION

An unsatisfactory feature of the classical theory even in its modified form was that it paid no attention to hemophilia the most important single coagulation defect. In hemophilic blood all the factors envisaged by the classical theory are present in normal amounts. In order to study the hemophilic defect it was necessary to ignore the theory and to study the subject from first principles.

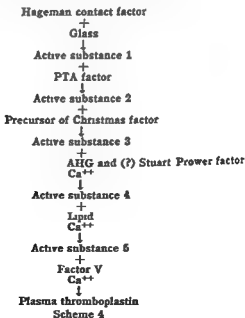
An early observation was that a fraction of normal plasma containing fibrinogen (Cohn fraction I) would correct the hemophilic abnormality *in vitro*. The correcting substance came to be called antihemophilic globulin (AHG). It would not work properly without platelets. Later it was found that a very powerful activator of prothrombin resembling prothrombinase could be formed from reagents derived entirely from normal blood. This substance is called plasma thromboplastin. At first it was thought that the substances necessary for plasma thromboplastin formation were AHG, platelets, factor VII, factor V and  $\text{Ca}^{++}$  but later work showed that this view was incorrect. Factor VII is not required for plasma thromboplastin formation but two factors the Christmas factor (plasma thromboplastin component or ITC or factor IX) and the Stuart Prower factor are required.

It is evident that the tissue and plasma prothrombin converting systems are similar and use certain components in common but they are different in their origin and in some of their requirements. For convenience the system involving tissue extracts or RVV is called the extrinsic thromboplastin system and that derived wholly from blood constituents is called the intrinsic thromboplastin system. The scheme can now be visualized as follows:



**THIRD MODIFICATION \ SURFACE CONTACT,  
PLASMA THROMBOPLASTIN ANTECEDENT (PTA)  
AND THE HAGEMAN FACTOR**

If whole normal blood is collected into glass and silicone treated tubes the blood in the silicone tube takes longer to clot than that in the glass



tube. Some reaction occurs on exposure of blood to glass. Evidence now suggests that two factors—the Hageman factor and the I PA factor—are concerned in the changes brought about by glass. Probably the Hageman factor is activated by contact. The activated substance then reacts with the PTA factor to produce another active product. This product is probably concerned in the activation of the Christmas factor.

To conclude this summary a sequence of possible reactions is given above. It should be realized that this scheme is highly hypothetical. The five active intermediate products proposed may have no basis in reality, and indeed the whole process may be found to involve a series of parallel reactions rather than a sequential chain of reactions.

## CLINICAL COAGULATION DEFECTS

### *Afibrinogenemia*

A brief glance at any of the coagulation schemes will show that there can be no clot when there is complete absence of fibrinogen from the blood. This particular defect is extremely rare.

The diagnosis of afibrinogenemia is easily made clinically. Plasma from such a patient does not clot upon the addition of thrombin, nor is there a visible precipitate with 25 per cent  $(\text{NH}_4)_2\text{SO}_4$  fractionation.

Typical cases have been described by Macfarlane [5] and Fernando and Dharmasena [6]. Neither of these patients was a severe bleeder. Both bled at the separation of the umbilical cord and from superficial abrasions. Neither had any hemarthroses or deep muscle hematomas. One (the patient of Fernando and Dharmasena) was admitted to hospital and operated on successfully for hemoperitoneum. The other died at the age of 28 from retroperitoneal bleeding.

Of great interest has been the mildness of the hemorrhagic diathesis in some of the reported cases. Some of the females have had normal menses, and the unusual complication of hemarthrosis does not seem to result in permanent crippling of the joint.

The generation of thromboplastin and thrombin has been found to be normal; it seems that only the synthesis of fibrinogen is defective. Gatlin and Borges [7] have shown that the half-life of fibrinogen infused into two such patients is approximately 4 days, a survival comparable to that in normal individuals.

**Genetics.** The two patients mentioned and many of the others recorded were the offspring of consanguineous marriages. Both sexes are affected, the defect being transmitted as an autosomal recessive trait. Patients exhibiting the clinical picture are considered to be homozygous for the defect. Multiple cases within a family have been reported [8]. Low levels of fibrinogen have been found in those presumed to be heterozygous.



carriers of the defect but the evidence that heterozygous carriers can thus be detected has been questioned [9]

### *Prothrombin Deficiency*

Prothrombin deficiency as a single defect is so rare as to be worth mention only. Biggs and Douglas [10] recorded a case of acquired deficiency in a man and Quick, Pesciotta and Hussey [11] have described three patients whose defect was probably prothrombin deficiency. Two of the patients were brothers who had normal parents, four normal siblings and normal maternal and paternal grandparents. One bled for 2 months following circumcision, bruised easily, bled after tooth extractions and had a probable hemarthrosis as well as hematuria. The second had suffered from posttraumatic bleeding, gastrointestinal bleeding, easy bruising and two episodes of bleeding into his calf muscles. The latter incident responded clinically to injections of vitamin K. Both boys had prolonged prothrombin times which in the case of the second could be shortened by administration of vitamin K. Both boys had normal levels of factor V and VII. No two-stage prothrombin time test was done to confirm the prothrombin deficiency. The third patient of Quick and coworkers was a 5-year old girl of an unrelated and entirely normal family. Mixing her plasma with that of the first boy gave no shortening of the prothrombin time.

### DEFECTS IN THE EXTRINSIC THROMBOPLASTIN SYSTEM

The study of the deficiency states of the extrinsic thromboplastin system began in one of the periods of relative clarity and simplicity of blood coagulation theory. In 1935 Quick [12] devised the one stage prothrombin time test. This test was based on the classical theory of blood coagulation (Scheme 1). It consists of adding a potent tissue extract to plasma and thereafter recalcifying. Whereas the clotting time of normal plasma in the presence of  $\text{Ca}^{++}$  alone may be 120 to 180 sec, the addition of tissue extract reduces the clotting time to 12 to 15 seconds. According to the classical theory this shortening of the clotting time is due to the accelerated conversion of prothrombin to thrombin. If the clotting time is reduced in this way to a minimum and if fibrinogen is present in adequate amounts, it follows from the classical theory that variations in clotting time are caused by variation in prothrombin content. Thus the one stage prothrombin time was used as a measure of prothrombin concentration.

It is now recognized that the one stage prothrombin time test practically never gives a measure of prothrombin itself. Prothrombin in normal blood is absent from serum because it is used up during clotting. It is also adsorbed from plasma by inorganic salts such as  $\text{BaSO}_4$ ,  $\text{BaCO}_3$  and  $\text{Al(OH)}_3$ . If to a plasma sample with a long one stage prothrombin

time is added either serum or adsorbed plasma there should be no shortening of the one stage prothrombin time providing the defect is pure prothrombin deficiency. Actually the clotting of practically all pathologic samples is accelerated by addition of normal serum or adsorbed plasma. Thus the defect in the patients is not of prothrombin itself but of some other factor in the thromboplastin system.

In addition prothrombin can be measured by a method which is independent of the factors other than prothrombin such as factor VII, Stuart Prower factor, factor V, etc. This is the two stage prothrombin time test. Its use also indicates that pure prothrombin deficiency is very rare. It is performed by adding tissue thromboplastin to platelet poor plasma and recalcifying. As thrombin is formed aliquots are taken at serial times for addition to tubes containing standard amounts of fibrinogen. From a previously constructed thrombin-fibrinogen calibration curve the amount of thrombin added can be deduced and the prothrombin in the initial sample calculated.

### *Factor V Deficiency*

In 1947 Owren [13] described a female patient whose plasma had a long one-stage prothrombin time. The clotting time was reduced practically to normal by adding adsorbed (prothrombin poor) plasma. Owren showed that the corrective factor was in the fraction of adsorbed plasma which precipitated between 33 to 50 per cent saturation with  $(\text{NH}_4)_2\text{SO}_4$ . Since it differed from all previously described factors he called it factor V. The factor is very unstable on storage in the presence of oxalate. It is not adsorbed by inorganic precipitates. It disappears during clotting and is therefore not present in serum. The plasma of a patient with factor V deficiency has a long one stage prothrombin time if RVV is used as thromboplastin.

**Clinical Findings.** Patients with this defect may be severe bleeders. Nose bleeding and massive bruises are common. After trauma the bleeding may be delayed for several hours but thereafter persists until healing is advanced. Scratches and small puncture wounds do not bleed excessively. Menorrhagia is a cause of serious anemia in females. Hemarthroses are uncommon. Other cases of factor V deficiency are referred to by Biggs and Macfarlane [1].

**Genetics.** The disease may occur in either sex. Sometimes more than one member of the family is affected. Relatives of affected members may show subclinical deficiencies of factor V. In other instances no other members of the family show any abnormality. The disease may occur in either sex and may be mild or severe in degree. Although sporadic cases occur a familial pattern is more common. Consanguinity has been noted in some instances but not in others. Persons with mild cases of factor V deficiency have been found to have 1 to 5 per cent of normal levels of

factor V, severely affected patients have demonstrated no detectable levels. Congenital deficiency of factor V has been reported in association with congenital heart disease [14] syndactylism [15] epidermolysis bullosa congenitalis [16] and decreased levels of prothrombin [15] and AHC [17]. There is as yet no general agreement on the mechanism of transmission. An excellent discussion of the inheritance of factor V deficiency and of other inherited clotting disorders is given by Ratnoff [18].

### *Factor VII Deficiency*

Only a few patients with a congenital bleeding tendency attributable to factor VII deficiency have been described. The defect is suspected if there is a long one-stage prothrombin time using brain extract as thromboplastin and if the clotting time by this test is reduced to near normal by the addition of normal serum. The uncomplicated nature of the defect is confirmed in modern terms if a normal blood thromboplastin forming system is demonstrated and if the one-stage prothrombin time using RVV as thromboplastin is also normal.

Factor VII is stable on storage and is adsorbed from serum by inorganic precipitates. On electrophoresis factor VII activity is found in the  $\beta$  globulin fraction.

**Clinical Findings.** The cases of Hicks [19], Ackroyd [20] and Pitney [21] are well described. Ackroyd's patient, a male, had severe epistaxis and two hemarthroses. He bled from small cuts in a rather peculiar way. The lesions would stop bleeding normally, but bleeding would restart in 3 to 4 hr. In later life he developed gastrointestinal bleeding. An operation on an ankle caused no undue bleeding. Hicks' patient, a female, bled at the time of eruption of her first teeth. She had epistaxes in childhood and later menorrhagia. Three major operations, two for umbilical hernia and one for appendicitis, were performed without undue bleeding. Pitney's patient, also a female, bled sufficiently to require transfusion after dental extractions. She bled excessively after eight deliveries. Dental extraction following plasma transfusion therapy was uneventful.

**Genetics.** The patients described above had no history of bleeding in other members of the family.

Deficiency of factor VII has been noted both on a sporadic and on a familial basis. In a family reported by Ratnoff the deficiency appeared to be inherited as a recessive trait and the heterozygous carrier could be detected. A similar situation was reported by Quick, Pisciotto and Hussey [11] who described a 3 year-old girl who had been a severe bleeder since birth. She was found to be factor VII-deficient. Her brother, parents and maternal grandparents were clinically normal, yet all but the maternal grandmother had mildly prolonged prothrombin times and were assumed to be heterozygous for the defect. Careful studies of factor VII-deficient families by Voss [22] and Dische [22a] also suggest the factor

VII mutant gene is inherited as an autosomal recessive or intermediate gene. Thus although the disease is congenital there is no general agreement about inheritance.

### Stuart Prower Defect

This abnormality has been defined in recent years from the work of Bergigel [23] Telfer Denson and Wright [24] Hougie Barrow and Graham [25] Graham Barrow and Hougie [26] Bachman Duckert Geiger Baer and Koller [27] and Denson [28]. There appear to be no specific distinguishing clinical features corresponding to this defect. The abnormality is associated with a long one stage prothrombin time when either brain extract or RVV is used as thromboplastin. The patient's

TABLE 35-3 DIAGNOSTIC TESTS FOR AFIBRINOGENEMIA AND FOR DEFICIENCIES OF PROTHROMBIN FACTOR V FACTOR VII AND THE STUART PROWER FACTOR

| Deficiency               | Clotting time | Two stage prothrombin test | Prothrombin test |               | Correcting effect of |             |
|--------------------------|---------------|----------------------------|------------------|---------------|----------------------|-------------|
|                          |               |                            | Brain            | RVV           | Id. bed plasma       | Sum         |
| Afibrinogenemia          | No clot       | Normal                     | No clot          | No clot       | Normal clot          | No clot     |
| Prothrombin deficiency   | Long          | Abnormal                   | May be normal    | May be normal | No clot              | No clot     |
| Factor V deficiency      | Long          | Normal                     | Long             | Long          | Normal clot          | No effect   |
| Factor VII deficiency    | Normal        | Normal                     | Long             | Normal        | No effect            | Normal clot |
| Stuart Prower deficiency | Long          | Normal                     | Long             | Long          | No effect            | Normal clot |

blood also shows an abnormality in intrinsic thromboplastin formation (see below).

**Genetics.** In the case of Hougie Barrow and Graham [25] the inheritance appeared to be through an autosomal recessive gene. In this family the members heterozygous for the defect were found to have subnormal levels of Stuart Prower factor. This was true also of the family described by Bachman et al. [27]. A pedigree for Stuart Prower factor deficiency has also been recently described by Roos Arkel Verloop and Jordan [29]. This family included a consanguineous marriage which produced 12 children, 6 of whom were bleeders. These 6 had levels of Stuart Prower factor between 5 to 6 per cent. The parents & 4 of the 6 asymptomatic children and several asymptomatic relatives were found to have subnormal levels of Stuart Prower factor and were assumed to be heterozygous. One female in this family was heterozygous for both Stuart Prower defect and alcaptonuria. Thus of the three families all who were

heterozygous were asymptomatic or nearly so and those homozygous for the defect were bleeders

The properties of factors V, VII and the Stuart Prower factor related to the extrinsic thromboplastin system are compared in Table 35 2 the diagnostic tests used to distinguish between the anomalies are given in Table 35 3

#### DEFECTS OF THE INTRINSIC THROMBOPLASTIN SYSTEM

##### *Hemophilia (Hemophilia A Antihemophilic Globulin Deficiency Factor VIII Deficiency)*

Because it is the best known and most common of the clotting defects hemophilia must occupy a central point of interest in any study of inherited disorders of blood coagulation The primary defect in this disease remains unsure The contemporary interpretation is that the blood of hemophilic patients lacks AHG a substance necessary for normal coagulation A minority view is that the defect is at least in part attributable to an inhibitory substance which depresses coagulation

Most of the evidence supporting the theory that an inhibitor is responsible for the hemophilic defect is indirect Thus the clotting of diluted and undiluted plasma is compared or comparisons are made of hemophilic and normal plasma as substrates in the thromboplastin generation test [30 31] These observations do not directly delineate an inhibitor An inhibitor in hemophilic plasma should destroy or mask AHG It is true that AHG disappears rapidly from the blood of treated hemophilic patients Various estimates assess the half life of AHG after treatment at 9 to 11 hr [12 33] Thus it is possible that hemophilic blood does contain an inhibitor but until the half life of AHG in normal blood is known there can be no certainty about this At present it has certainly proved of practical value to treat hemophilic patients as if the main defect is AHG deficiency

Probably more is known of the nature of AHG than of any of the other clotting factors except  $\text{Ca}^{++}$  The substance lacking from hemophilic blood is very unstable Normal human blood collected for transfusion may lose much of its activity in 6 hr and the activity may be reduced to 30 per cent of the original in 24 hr The activity is better preserved if plasma is separated soon after collection and frozen at  $-20^\circ\text{C}$  but even at this temperature some activity is lost Activity is usually also lost during freeze drying During coagulation AHG disappears This utilization or destruction during clotting may be in part responsible for the loss of activity during the ordinary collection of blood for transfusion because in collection a minimum amount of coagulation is usual and this may be sufficient to dispose of much AHG activity Careful attention to mixing during collection seems to reduce losses [34]

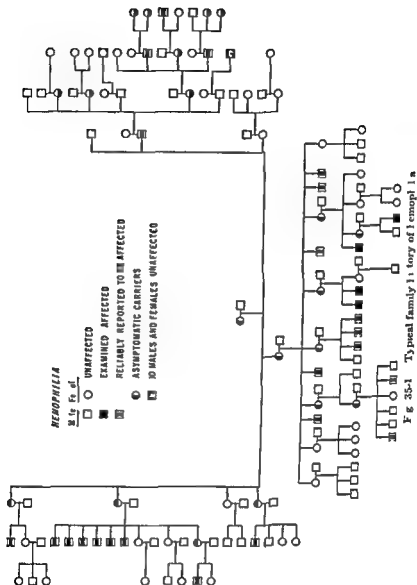
Fractionation by several different methods shows that AHG precipitates with the fibrinogen fraction of plasma. No separation of activity from human fibrinogen has been achieved without gross loss of activity. On the other hand the blood of patients with afibrinogenemia contains AHG [36]. Electrophoresis of plasma from such a patient has shown that AHG separates with the  $\beta$  globulin fraction [35].

AHG separated with fibrinogen has a narrow pH range of relative stability. It is destroyed by heating and by storage at room temperature or at 0°C. AHG is not adsorbed by inorganic precipitates such as  $\text{BaSO}_4$  and  $\text{Ca}_3(\text{PO}_4)_2$ .

**Clinical Features** The majority of hemophilic patients are males. The disease in its classical form usually declares itself in early childhood when mobility exposes the child to increasing minor trauma. Bruises disproportionate to the injury appear and disabling joint swellings may follow. The painful joint confines the patient to bed and the muscles waste. During convalescence the joints may be poorly protected and hemarthroses commonly recur. A self-perpetuating cycle of hemarthroses and wasting is initiated until by adolescence the young man may have already acquired deformed and weakened limbs. Equally disabling and often more dangerous bleeding into muscles, particularly the retroperitoneal muscles, may occur at any time. Hematuria, gastrointestinal bleeding, and epistaxis are common. Trauma such as tongue biting, small cuts, and dental extractions all give rise to troublesome and prolonged bleeding which may require transfusion.

A characteristic feature of the bleeding in hemophilic patients is that it occurs in phases. The patient in a good phase may live almost normally for months and sometimes years, surmounting many minor injuries with little inconvenience. At other times massive and apparently spontaneous bruises appear. The least injury may produce hemarthroses and further lesions appear while the patient is resting in bed to recover from the first. No consistent changes in the hemostatic mechanism have been found to account for these variations.

Another feature of hemophilic bleeding is its delayed onset. If an operation is carried out on an untreated hemophilic patient the surgeon is often pleasantly surprised by the freedom from bleeding at the time of operation. This happy state is often interrupted 6 to 8 hr later by a steady, slow, and persistent flow of blood. The surgeon returns to search for bleeding points and usually some bleeding vessel is found and ligated. Such operations are often unsuccessful and a continuing battle against a leaking sieve may continue with constant blood replacement until the much delayed healing finally happens or death occurs. Even the treated patient is susceptible to secondary hemorrhage, usually from apparently irrelevant small vessels. A single tooth socket may bleed for 4 to 6 weeks if no specific treatment is given.



This is the general clinical picture of bleeding in the severely affected patient but many hemophilic patients have a much milder version of the disease. In some the defect is disclosed only by serious injury, when unexpected bleeding stimulates a laboratory study.

**Genetics.** Hemophilia is inherited as a sex-linked recessive characteristic. A typical family record is shown in Fig. 35.1. The vast majority of families with more than one affected member show a pattern consistent with this interpretation. Exceptional families in which an occasional female has a mild bleeding tendency are often interpreted quite satisfactorily on the hypothesis that the gene is incompletely recessive.

Several cases of apparently true hemophilia in females have been reported. Three sisters who were the offspring of a consanguineous marriage in a hemophilic family were reported by Merskey [36]. They suffered from post wound and post tooth extraction bleeding, hematuria and gastrointestinal bleeding but survived childbirth and bore hemophilic sons. The female reported by Iralis [37] was born of unrelated parents who were a hemophiliac and a hemophilia carrier respectively. A recent case of sporadic AHG deficiency in a 6-year-old white female has been reported by Quick and Hussey [38]. A colony of hemophilic dogs which has been maintained at the University of North Carolina for several years has produced hemophilic females following the crossing of a hemophilic male and a carrier female [39]. Families studied often show an apparent excess of affected males [40] but this disproportion is probably due to bias in selection. No family is studied unless at least one affected male has been discovered and thus families of carriers fortunate enough to have all normal children are omitted.

Nearly half the families studied have only one affected male. There are two interpretations for this phenomenon. Either the members of the family are ignorant about affected ancestors and distant cousins or the disease has arisen anew by mutation. There are very few records sufficiently complete for certain distinction between the two possibilities. Calculations based on the life expectation of hemophilic males which is variously estimated at 16 to 22 years indicate that mutation must be fairly frequent because there is little obvious tendency for the disease to decrease.

**Christmas Disease (Hemophilia B, Factor IX Deficiency, PTC Deficiency, Hemophilia B, Factor IX Deficiency)**

This condition resembles hemophilia so closely that until recently the two abnormalities were not separated. The clinical manifestations of the two are identical as is the mode of inheritance. The conditions are distinguished because a mixture of equal parts of blood from patients with hemophilia and Christmas disease clots normally whereas the separate samples clot abnormally. The two defects are caused by defi-



*ciency of different factors [41, 43] Little is known of the nature of the factors*

**Clinical Features** Little needs to be said about the clinical features of Christmas disease since they are identical with those of hemophilia. Among the senior author's patients there are more mild cases of Christmas disease than of hemophilia. This may be a local phenomenon. A large proportion of the mildly affected patients are derived from one area. Although there is no evidence for relationship between the different families, it seems very probable that there may be common ancestors. It should not be thought on this account that Christmas disease is a milder defect than hemophilia. Some Christmas disease patients are very severely affected.

A major and unique contribution to the study of Christmas disease comes from Switzerland [43, 44]. The geographic, social and religious organization of residents in the Tenna Valley is such that relationship of a family can be traced back with considerable certainty for 11 generations. In this family 3,072 members, including 55 bleeders, have been recorded. Moor-Jankowski [43, 44] and his colleagues have brought the records of this family up to date and have personally studied 10 bleeders. All these patients had Christmas disease and Christmas factor levels of only 2.5 to 6 per cent of normal. Thus from the laboratory point of view there is great uniformity in the expression of the disease. Nonetheless there were marked differences among the different bleeders. Some were severely affected from early childhood; others had no symptoms for many years. The authors also noted a general tendency for the bleeding to be less severe with advancing years. The average life expectancy of affected members is 22 years.

**Genetics** The inheritance of Christmas disease is the same as that of hemophilia, as a sex-linked recessive. The study of the Tenna Valley family in Switzerland adds much to detailed understanding [43, 44]. This family has one case of Christmas disease that must be regarded as arising through mutation. A strange finding in this family is that female carriers have significantly more children than the normal females. In this family there is no natural tendency of the disease to die out even without mutation. The decreased number of offspring of affected males is almost exactly balanced by the increased fertility of the carriers. This record is invaluable because it provides a really good basis for testing hypotheses about inheritance in an exceptionally well recorded and studied single family.

**Surface Contact (Plasma Thromboplastin Antecedent (PTA) Deficiency and the Hageman Defect)**

The effect on clotting of contact with glass is well recognized. This phenomenon suggests that glass brings about some alteration in the

blood Recent studies have connected this phenomenon with two pathologic blood conditions PTA deficiency and the Hageman defect, [47-51] The 2 defects are currently considered together

**PTA Deficiency** In 1953 Rosenthal Dreskin and Rosenthal [46] described a new hemorrhagic state which appears to be a result of PTA deficiency and is inherited as an autosomal dominant characteristic All four patients with this syndrome that have been seen in the senior author's clinic have been Jewish This observation may have no significance but if there is a true association with Jewish ancestry the variable distribution of the disease in different parts of the world would be explicable The disease is for example common in New York but uncommon in England [40]

Patients with PTA deficiency are mostly rather mild bleeders In two female patients of the authors menorrhagia necessitated hysterectomy All have had relatively marked bruising tendency and all have bled after dental extractions or operations Occasionally more severely affected patients have had hemarthroses or muscle hematomas

**The Hageman Defect** [47] This syndrome is a strange and subversive phenomenon in the blood clotting field The eponym derived from the name of the first patient studied Patients with no hemostatic defect clinically have occasionally been found with gross laboratory abnormalities The absence of clinical defect has been confirmed in all cases by multiple uneventful surgical operations The condition therefore is readily distinguished from PTA deficiency in which there is bleeding The abnormality appears to be familial but the mode of inheritance is unclear

**Physiologic Interpretations** It is convenient to consider these two abnormalities together because the laboratory results are similar Blood samples from patients with either syndrome may have a long clotting time and an abnormal prothrombin consumption In both abnormalities the thromboplastin generation test shows an unusual pattern but the results are often abnormal only if both serum and adsorbed plasma are derived from the patient In some cases a slight apparent deficiency of Christmas factor occurs In others the thromboplastin generation test gives entirely normal results

Conflicting results have emerged from many aspects of laboratory testing in these cases Fractionation work has not revealed a specific PTA fraction with any clear definition Samples initially apparently deficient in PTA factor may approach normality on storage Mixture experiments may give confusing results samples from the same patient may be considered to show PTA deficiency or Hageman defect on different occasions Many of these difficulties can be attributed to the nature of the underlying defect

In both these conditions there appears to be a defect in ability to react

to glass surface. Thus depending on the degree of glass exposure of samples being tested the results will vary. From the observations of Biggs et al. [45] it seems that if samples are collected in silicone treated apparatus a glass exposed PTA deficient sample will correct the defect of a silicone-stored sample from a Hageman defect patient but will not correct the abnormality of a silicone-collected PTA deficient specimen. In the reverse experiment a glass-exposed sample from a Hageman defect patient will not correct the abnormality of a silicone-collected PTA deficient specimen. These experiments suggest that the Hageman factor is the one which reacts to contact with glass and that the product of this activation reacts with the PTA factor.

The apparent Christmas factor deficiency which may be encountered in these cases is probably due to slow activation of a precursor of the Christmas factor. If a mixture of PTA deficient and Christmas disease serums is allowed to stand before testing the abnormality may disappear [45]. It presumably takes some time for the PTA factor in the Christmas disease serum to activate the precursor of Christmas factor in the PTA deficient serum.

#### MULTIPLE FACTOR DEFECTS

##### *von Willebrand's Disease*

This disease would not previously have been included among the constitutional coagulation defects because the main abnormality has always been considered a failure of capillary function. It is now recognized that many patients with this syndrome are also deficient in AHG. A new name for this disease, vascular hemophilia, has recently gained favor but since the original family described by von Willebrand [52] had AHG deficiency there would seem to be little ground for multiplication of nomenclature.

In the more severely affected subjects there is evidence for a dual defect, one of the clotting mechanism and the other of the capillaries. Severely affected patients often have a level of AHG which is in the range seen in the blood in mild hemophilia but this association of defects is by no means found in all cases. Nothing is known of the intimate nature of the capillary defect. One possible explanation is that abnormality of the small vessels produces AHG deficiency when the vessel anomaly is sufficiently severe. Perhaps such vessels lead to rapid utilization of AHG, or perhaps AHG is made in the normal vascular endothelium.

**Clinical Features.** Patients with this condition bruise easily. They have frequent and often severe epistaxes. Females may have severe menorrhagia which may require transfusion. There may be bleeding from the gums and there is bleeding always after dental extraction. Small cuts bleed freely but the blood flow is stopped easily and usually permanently by pressure. Surgery has proved surprisingly uneventful. There is

usually some excessive bleeding, but the persistent and uncontrollable bleeding of the hemophilic patient is absent.

**Genetics** The condition is inherited as an autosomal dominant trait. A typical family tree is given in Fig. 35.2. In mildly affected patients the level of AHG may be normal. Nilsson, Blombäck and Francken [53] record a family in which a low AHG level occurred in association with von Willebrand's disease. Study of clinically unaffected members of the

# VON WILLEBRAND'S DISEASE

M F I

- ○ NORMAL
- ● RELIABLY REPORTED TO BE AFFECTED
- ● EXAMINED AFFECTED
- ◇ SEX UNKNOWN
- ○ 4 MALES AND FEMALES NORMAL

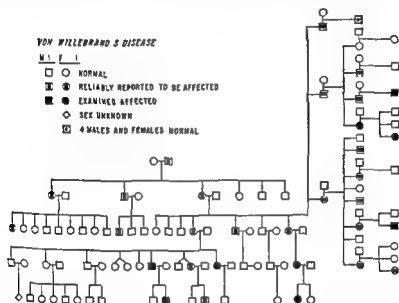


Fig. 35.2. Family history illustrating the inheritance in von Willebrand's disease.

family showed that some of them had low AHG levels. There is thus the possibility that the two features (AHG level and capillary function) are inherited separately and that the combination gives rise to severe examples of disease. Such severely affected patients are more frequently studied than less serious bleeders. This possibility was suggested by Pitney [54]. If this is the case, this variety of AHG deficiency is inherited as an autosomal dominant characteristic.

## *Dicoumarol Intoxication*

A most informative and relevant abnormality (although not an inherited disorder) is that caused by the dicoumarol group of drugs. The type of clotting anomaly depends upon the duration of treatment and to some extent on the drug used. A patient who has been treated for more than a week and whose level of prothrombin time is within the therapeutic range usually shows a pattern as follows:

| <i>Factor</i>        | <i>Percentage of Normal</i> |
|----------------------|-----------------------------|
| Prothrombin          | 40-70                       |
| Factor VII           | 5-10                        |
| Christmas factor     | 10-50                       |
| Stuart Prower factor | 10-50                       |

In addition there may be deficiency of yet another coagulation factor, which is at present ill defined. The level of Christmas factor and Stuart Prower factor depends upon the extent of the defect. Exactly the same pattern is found in vitamin K deficiency and in some congenital cases with multiple clotting defects [55-56]. These observations suggest that there must be some common pathway for the manufacture of these factors or that the defect represents an interference with some factor on which all the others depend. The blood of these patients may show confusing results with conventional tests. The clotting time is usually normal because none of the factors is reduced to a rate-limiting degree. Thromboplastin generation is often quite abnormal when the patient's serum replaces normal serum. Mixture experiments may give confusing results unless specifically defective samples are used.

Other reported anomalies include combined deficiency of AHG and factor V [17-58] or factor VII and Stuart Prower factor [29].

## LABORATORY DIAGNOSIS OF CLOTTING FACTOR DEFECTS

The clinical tests of clotting for the most part relate to measurements of the components of the internal thromboplastin system. Some of these will be reviewed here in order to indicate the extent of the current experimental limitations in the coagulation field.

### *Clotting Time*

In this test a measured quantity of blood is put into a glass tube and the time until solidification is complete is recorded. The details of technique vary from center to center. Usually four tests are done and average results reported. The test is entirely nonspecific; any defect at any stage may influence the results. The test has been modified for quantitative measurement of a specific factor by taking the blood of a patient with a known defect, e.g., hemophilia, and adding to this the blood of another patient. If the clotting time of the mixture is shorter than that of the standard patient then the undiagnosed patient has some other defect. By adding different known proportions some quantitative estimate can be made. The use of the test in this way is open to much criticism and in practice is most inaccurate. The test will for example record normal results with as little as 1 to 5 per cent of AHG in a sample of hemophilic blood. Moreover the practice rests on the assumption that the defect in the standard plasma is accurately known. This is certainly not often

true For example before the recognition of Christmas disease the random selection of a hemophilic plasma as a standard would not infrequently have resulted in the choice of a sample from a patient with Christmas disease

### *Prothrombin Consumption*

During normal coagulation prothrombin is converted to thrombin and little remains in the serum If the intrinsic plasma thromboplastin forming system is defective then much prothrombin is found in serum The test consists in making an empirical assessment of the relative amount of prothrombin present in the serum The test is much more sensitive than the clotting time to minor deviations from normal The test is in itself quite nonspecific It gives no information about what is wrong but merely records abnormality It has been used with standard blood samples to define new defects in the manner described for the clotting time test

### *Thromboplastin Generation*

In this test reagents prepared from normal plasma when mixed together can be shown to generate a powerful thromboplastic agent in the presence of calcium ions [62] The reagents required are  $\text{Al}(\text{OH})_3$  or  $\text{BaSO}_4$  treated plasma serum and platelets To analyze the reactions involved it is necessary to consider the factors contained in this complicated mixture

**$\text{Al}(\text{OH})_3$  or  $\text{BaSO}_4$  treated Plasma** This contains AHG and factor V Experiments in which one or the other of these factors is excluded from the system have shown that both are essential

**Serum** Serum contains factor VII Christmas factor and the Stuart Prower factor Experiments this time with serum from patients who lack one or the other of these factors have shown that Christmas factor and the Stuart Prower factor are required but that factor VIII is not essential

**Platelets** Platelets can be replaced in the thromboplastin generation test by preparations of brain phospholipids made according to the method of Folch [63] With this information there have been many attempts to identify a chemically definable fraction as the one active substance Some workers have found that attempts at purification are accompanied by loss of activity and have concluded that a single active substance cannot be located [64] Others have implicated phosphatidylethanolamine [65-66] and phosphatidylserine [66-68] as the active compound Recent work suggests that several phosphatides may be required [68] a suggestion which would account for the loss of over all activity during purification

The thromboplastin generation test is used as a diagnostic aid In diagnosis it is used to distinguish between hemophilia and Christmas disease by making mixture reagents from normal blood and a patient's blood The general plan of such a diagnostic test is shown in Table 35-4 This test does not require a standard abnormal sample to define the

abnormality but it does rest on the assumption (probably false) that all the main coagulation factors are known

TABLE 35.4 THROMBOPLASTIN GENERATION TEST USED FOR DIAGNOSIS OF HEMOPHILIA AND CHRISTMAS DISEASE IN PATIENTS WHOSE PLASMA HAS A NORMAL ONE STAGE PROTHROMBIN TIME

| Source of $Al(OH)_3$<br>treated plasma | Source of<br>serum | Source of<br>platelets or<br>phospholipid | Results      |                      |
|--|--------------------|---|--------------|----------------------|
|  |                    |   | Hemophilia   | Christmas<br>disease |
| Normal                                 | Normal             | Normal                                    | Control test |                      |
| Normal                                 | Patient            | Normal                                    | Normal       | Abnormal             |
| Patient                                | Normal             | Normal                                    | Abnormal     | Normal               |
| Patient                                | Patient            | Normal                                    | Abnormal     | Abnormal             |

Both factor V and Stuart Prower factors are required for blood thromboplastin formation deficiencies of these two factors are distinguished by the thromboplastin generation test as shown in Table 35.5 In tests using these samples phospholipid [69] should be used in place of platelets because platelets adsorb factor V from normal plasma and the use of normal platelets may mask the defect

TABLE 35.5 THROMBOPLASTIN GENERATION TEST RESULTS FOR PATIENTS WITH FACTOR V OR STUART-PROWER DEFECT

| Source of $Al(OH)_3$<br>treated plasma | Source of serum | Results                |                         |
|--|-----------------|------------------------|-------------------------|
|  |                 | Factor V<br>deficiency | Stuart Prower<br>defect |
| Normal                                 | Normal          | Control test           |                         |
| Normal                                 | Patient         | Normal                 | Abnormal                |
| Patient                                | Normal          | Abnormal               | Normal                  |
| Patient                                | Patient         | Abnormal               | Abnormal                |

The one-stage prothrombin time test carried out on the plasma of these patients gives abnormal results

Factor VII deficiency causes no blood thromboplastin defect thus clotting time prothrombin consumption index and thromboplastin generation test all give normal results

The thromboplastin generation test can also be adapted to study the physiology of blood thromboplastin formation Thus Biggs Douglas and Macfarlane [70] showed that if AHG serum and calcium are in

cubated together an active intermediate product of coagulation is formed. The interaction of this product with platelets produces an active particulate substance which can be sedimented by centrifuging [71]. Biggs and Bidwell [72] showed that the final product of thromboplastin formation is unstable but can be reactivated by the addition of factor V. Hougie and his coworkers [25] have shown that the Stuart Prower factor is probably concerned together with Christmas factor in reaction with AHG.

*Modification of the Thromboplastin Generation Test for the Assay of AHG [32]*

A method for the quantitative assay of AHG can be devised if it is assumed that AHG affects quantitatively the yield of active thromboplastin and that preparations which give the same minimum clotting time by this test have the same activity. Using this method it has been possible to predict from blood levels of AHG the severity of bleeding to be expected. Thus if a patient has more than 5 per cent of a normal level of AHG he will be very mildly affected. Again if levels below 25 per cent of normal are achieved by therapy the bleeding will not be controlled adequately after surgical treatment. From these practical results it is reasonable to assume that the test gives some measure of the hemophilic defect [32]. It should be emphasized that a common sense assessment of success or failure in practice is the only way of judging whether or not assumptions about a quantitative method are reasonable.

The election of techniques described is not comprehensive nor are they necessarily the best tests. The main purpose of this review is to bring into focus the limitations of this whole field of study. In Table 35.6 the methods have been related to the theory of blood coagulation. It will be seen that even assays for specific factors may be affected by many variables and that the factors which affect the cruder screening tests such as the clotting time and prothrombin consumption tests, are innumerable. In the more specific assays attempts are made to eliminate undesirable variables by using standard plasma samples or by supplying irrelevant factors at high concentration. Despite this care most coagulation tests rank in accuracy and reliability more with rather crude biologic assays than with chemical analyses.

## BLOOD COAGULATION AND HEMOSTASIS

There now remains the question of the relationship between hemostasis and blood coagulation. The conventional view of hemostasis [73] is of a dual mechanism involving platelet, capillary and arteriolar function on the one hand and blood coagulation on the other. A small injury ceases to bleed because the vessels are contracted and plugged by plate



TABLE 3.6 APPROXIMATE RELATIONSHIP OF TESTS USED IN THE DIAGNOSIS OF COAGULATION DEFECTS TO THE STAGES OF BLOOD COAGULATION WHICH AFFECT THEM

| Stages of the extrinsic thromboplastin system using mammalian tissue extract (thromboplastin)   | Tests applicable to the extrinsic system  | Tests applicable to both extrinsic and intrinsic systems  | Tests applicable to the intrinsic system  | Stages of the intrinsic thromboplastin system  |
|---|---|---|---|--|
| Brain extract + factor V + factor VIII<br>+ Stuart Prower factor + $\text{CaCl}_2$<br>↓<br>Prothrombinase + prothrombin<br>+ $\text{CaCl}_2$<br>↓<br>Thrombin + fibrinogen<br>↓<br>Fibrin | O + 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 | T-est R<br>↓<br>T-est L<br>↓<br>T-est M<br>↓<br>T-est N<br>↓<br>T-est O<br>↓<br>T-est P<br>↓<br>T-est Q<br>↓<br>T-est R<br>↓<br>T-est S<br>↓<br>T-est T<br>↓<br>T-est U<br>↓<br>T-est V<br>↓<br>T-est W<br>↓<br>T-est X<br>↓<br>T-est Y<br>↓<br>T-est Z | T-est A<br>↓<br>T-est B<br>↓<br>T-est C<br>↓<br>T-est D<br>↓<br>T-est E<br>↓<br>T-est F<br>↓<br>T-est G<br>↓<br>T-est H<br>↓<br>T-est I<br>↓<br>T-est J<br>↓<br>T-est K<br>↓<br>T-est L<br>↓<br>T-est M<br>↓<br>T-est N<br>↓<br>T-est O<br>↓<br>T-est P<br>↓<br>T-est Q<br>↓<br>T-est R<br>↓<br>T-est S<br>↓<br>T-est T<br>↓<br>T-est U<br>↓<br>T-est V<br>↓<br>T-est W<br>↓<br>T-est X<br>↓<br>T-est Y<br>↓<br>T-est Z | Hageman contact factor + glass<br>↓<br>Active substance 1 + PT A factor<br>↓<br>Active substance 2 + precursor of Christmas factor<br>↓<br>Active substance 3 + AHC and (7) Stuart Prower factor + $\text{CaCl}_2$<br>↓<br>Active substance 4 + lipid + $\text{CaCl}_2$<br>↓<br>Active substance 5 + factor V + $\text{CaCl}_2$<br>↓<br>Plasma thromboplastin + prothrombin + $\text{CaCl}_2$<br>↓<br>Thrombin + fibrinogen<br>↓<br>Fibrin |

lets. After a lapse of time the vessels relax and bleeding is not resumed because the vascular lumen is filled by clot. For larger injuries vascular contraction cannot stem the flow and ligature or pressure must arrest the bleeding and allow time for blood coagulation. There is no reason to suppose that this view is not substantially correct. A detailed analysis of bleeding associated with single-factor defects may suggest whether or not hemostasis may be achieved by one of the several mechanisms available and whether or not the mechanisms are different in different tissues. A study of some inconsistencies may also reveal new fields for exploration.

By comparing factor VII deficiency with hemophilia or Christmas disease the relative importance of the intrinsic and extrinsic thromboplastin systems may be assessed. The first point of interest is that two of the factor VII-deficient patients described had operations with no very excessive bleeding, whereas in hemophilic patients operations are notoriously dangerous. This observation if confirmed for other cases of factor VII deficiency suggests that the intrinsic (blood) thromboplastin system is the more important for sustained hemostasis. This conclusion is also borne out by a more detailed study of hemophilic bleeding. Hemophilic patients are very liable to secondary hemorrhage. Even a treated patient will start to bleed if the treatment is inadequately maintained. One large dose of AHG to a hemophilic patient will stop bleeding for 24 to 48 hr. This observation suggests that normal hemostasis involves the continued renewal of clot and that the intrinsic thromboplastin mechanism is of primary importance for the process.

If bleeding at different sites is considered it becomes clear that hemarthroses are associated with severe hemostatic defects whatever the cause. Hemarthroses are usually linked with hemophilia because hemophilia is the most common serious hemostatic defect but mildly affected hemophilic patients do not have hemarthroses and severely affected patients with extrinsic thromboplastin defects do. Hematuria can occur in any defect. Persistent oozing from small skin injuries and from the gums is predominantly a feature of capillary abnormalities. Deep muscle hematomas are much more common in hemophilia than in any other disease. It may be that the muscle tissue is a poor source of thromboplastin and that muscle hemostasis normally depends very little on the extrinsic system. The peculiar observation of Ackroyd that a factor VII-deficient patient tended to have recurrent hemorrhage from trivial superficial injuries and the capillary defect (indicated by a long bleeding time) which may occur in patients with vitamin K deficiency and after excessive dosage of dicoumarol suggest that the extrinsic thromboplastin system may have something to do with the maintenance of capillary function.

There are certain interesting inconsistencies between laboratory results and hemostatic function. The most obvious of these is the absence of

abnormal bleeding in patients with the Hageman defect. Workers are so familiar with the usual dire significance of a long clotting time that a syndrome in which abnormality in this test does not herald a severe hemostatic defect is a surprise. The only reasonable interpretation is that in the body the stimulus initiating coagulation is not a surface which resembles glass in any way. The glass surface may perhaps by pass or replace the natural stimulus to clotting. The failure of the glass mechanism would not necessarily be associated with any in vitro hemostatic defect. One might reason that it should be possible to find patients who have clinical hemorrhagic states but in whom the natural stimulus to clotting is defective. Such patients should have no clotting abnormality if their blood is tested in glass tubes. Coagulation specialists often encounter patients who give a definite history of bleeding but whose laboratory tests are negative. Some of these cases may have the postulated defect but proof is at present impossible.

Another and much more fundamental difficulty concerns the coagulation defect in afibrinogenemic patients. The bleeding tendency in these patients should be the most severe of all. In fact the bleeding is usually less pronounced than in a severely affected hemophilic patient. This is a disturbing paradox to which at present there is no solution. It is difficult to understand why slow and defective clotting should be more dangerous than no clotting at all. Sooner or later this problem must be faced. It is possible for example that AHG is an essential component for healing and that one of the main defects in hemophilia is a delay in healing. It is also possible that the congenital absence of fibrinogen stimulates a particularly good mechanism of vascular contraction and platelet agglutination and that this mechanism can to some extent replace the functions of coagulation.

Another perplexing problem concerns the different types of bleeding in patients with hemophilia and von Willebrand's disease. Although patients with von Willebrand's disease may have low AHG levels they do not bleed in the same way as hemophilic patient. The patients with von Willebrand's disease have epistaxis and the females have menorrhagia; they bruise easily but do not have massive muscle hematomas. Bleeding after dental extraction is immediate and readily controlled by pressure. Postoperative hemorrhage is rarely disastrous. The characteristic hemophilic hemorrhage is a slow persistent ooze not controlled by pressure. Hemophilic patients do have deep muscle hematomas and postoperative bleeding is often disastrous. There is at present no explanation for this discrepancy. It is possible of course that the AHG deficiency of von Willebrand's disease is due to a rapid utilization of AHG by poor capillaries and it is also possible that hemophilic patients often have some as yet ill defined inhibitory factor which reinforces the underlying AHG defect.

## SUMMARY

1 Interpreted as molecular diseases the coagulation defects are sadly lacking in definition. There is no biochemical definition by current techniques but only alignment with empirical schemata. There is also no definition from clinical or metabolic features of the patients.

2 In due course it may be possible to interpret coagulation defects in terms of deficiency of definite chemical species or reaction sequences. At present no single chemical substance (except calcium) has been identified. The present study of the role of phospholipids is of particular importance; by continuing in this direction it may become possible for the first time to define a coagulation factor chemically.

3 Complex events precede the formation of fibrin from fibrinogen. This reaction is controlled by thrombin which in turn is derived from prothrombin by the combined action of calcium and plasma thromboplastin or prothrombinase.

4 Prothrombinase activity resides either in an external system composed of tissue extract and calcium or in an internal system derived from the platelets, plasma coagulation factors and  $\text{Ca}^{++}$ . Each of these prothrombinase systems is complex in itself and contains several factors necessary for prothrombinase activity.

5 Clinical hereditary coagulation defects may result from deficiency of any one of the several components of the clotting mechanism. Fibrinogenemia is one of these with mild clinical implications. It is an autosomal recessive disorder.

6 Defects in the extrinsic thromboplastin system include (a) factor V deficiency disease which may be a severe bleeding disorder and which has an uncertain inheritance pattern; (b) factor VII deficiency also with severe bleeding tendency and probably autosomal recessive inheritance; (c) Stuart Prower defect a disorder with clinical findings identical to those of factor VII deficiency and with autosomal recessive inheritance.

7 Defects of the intrinsic thromboplastin system include (a) classical hemophilia which is a result of hereditary absence of the AHG component and has recessive sex-linked inheritance; (b) Christmas disease resulting from deficiency of plasma thromboplastin component indistinguishable clinically from classical hemophilia and having also a sex-linked recessive inheritance; (c) Hageman factor defect which has no clinical manifestations; and (d) plasma thromboplastin antecedent a mild bleeding disorder with an uncertain inheritance pattern.

8 Occasionally multiple factor defects are encountered. Among these is von Willebrand's disease which may appear as a dual defect of the capillaries and the clotting mechanism. The bleeding tendency is mild. It is usually inherited as an autosomal dominant trait.

9 No clarity has emerged from the study of blood coagulation factors

in relation to hemostasis but the shadowy outline emerging suggests that hemostasis may be achieved through several partly independent but connected mechanisms. If one mechanism fails, others may in part replace it.

10 At present it must be admitted that there is an atmosphere of fantasy and improbability about the whole subject. Future workers may well view the present elaborate superstructure of hypothetical factors and hypothetically interpreted tests with the tolerance which we now afford to those who thought blood letting was a valuable therapeutic agency.

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## **Part Ten**

### **Diseases Manifest Primarily in Renal Tubular Transport**

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## Chapter 36

# Familial Hypophosphatemia and Vitamin D-resistant Rickets

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### DEFINITION

Familial hypophosphatemia and vitamin D-resistant rickets may be defined as a specific disorder characterized by (1) hypophosphatemia associated with decreased renal tubular reabsorption of inorganic phosphate (2) familial occurrence the mode of inheritance being most probably sex linked dominant (3) the presence in some but not all affected persons of rickets or osteomalacia which does not respond to the usual doses of vitamin D (4) the absence of other related abnormalities

This definition excludes conditions which may share the same clinical or physiologic features or biochemical defects such as sporadic (non familial) vitamin D-resistant rickets associated with hypophosphatemia and familial vitamin D-resistant rickets associated with normal or high serum concentrations of inorganic phosphate Patients with multiple renal tubular defects such as the Fanconi syndrome or renal tubular acidosis appear to have similar but more extensive abnormalities

Regarding the limited group defined above as a separate entity has several advantages The clear-cut genetic pattern allows one to identify a substantial number of persons who presumably have the same fundamental physiologic and biochemical abnormality Furthermore the presence of a single gross defect in renal tubular function simplifies the analysis Clarification of a single aberrant mechanism is a logical step in the study of the multiple defects of related conditions

### HISTORICAL DEVELOPMENT

The growth of understanding of this disorder has proceeded along clinical physiologic and genetic pathways Rickets which failed to

respond to usual doses of vitamin D was recognized only after vitamin D-deficiency rickets had been largely eliminated by prophylaxis. In such a patient Albright, Butler and Bloomberg [1] in 1937 first showed that very large doses of vitamin D would bring about healing of the rickets. That the hypophosphatemia was due either primarily or secondarily to decreased renal tubular reabsorption of phosphate was first recognized by Robertson, Harris and McCune [2] in 1942.

The first description of familial occurrence of vitamin D-resistant rickets with hypophosphatemia was in 1941 by Christensen [3] who reported a mother and her son and daughter with typical features of the disease. Earlier instances of familial occurrence are probable [4]. On the basis of skeletal manifestations several workers proposed an autosomal dominant mode of inheritance with variable penetrance [5, 6]. However, Winters and his collaborators [7-9] were able to show that the occurrence of hypophosphatemia in involved families follows a sex-linked dominant mode of inheritance with virtually complete penetrance. They worked on the assumption that the basic disorder lies in the abnormal renal excretion of inorganic phosphate with resulting hypophosphatemia and that the rickets and osteomalacia are secondary manifestations.

## CLINICAL AND RADIOLOGIC FINDINGS

The mildest abnormality is purely biochemical. This is hypophosphatemia (see below under Hypophosphatemia for criteria for hypophosphatemia) without clinical manifestations other than a slight decrease in height when compared with normophosphatemic siblings. This is the most common finding in kindreds with the disorder [7-9].

In hypophosphatemic adults the varying degrees of deformities due to rickets in childhood constitute more serious disturbances. These include bowing of the legs and shortening of stature usually without evidence of continuing active bone disease. A few affected adults have evidence of continuing osteomalacia as judged by the presence of pseudofractures and an elevated serum alkaline phosphatase level. These latter changes revert towards normal upon administration of massive doses of vitamin D.

In affected children with rickets the disease is usually first recognized when the child begins to walk, but the history or x-ray examination often reveals abnormalities dating to the first year of life such as deformities of the skull, late dentition and 'sitting' deformities of the legs. These children usually have received prophylactic doses of vitamin D and have failed to respond to the doses given to cure what was initially thought to be vitamin D-deficiency rickets. Healing of the active rickets occurs upon treatment with very large doses of vitamin D (usually 100,000 I.U. or more per day). Permanent deformities and shortened stature often are present. In adults and in children the use of the massive doses of vitamin D required for healing frequently results in the appearance of

vitamin D intoxication with discontinuance of the vitamin the rickets usually becomes active again. Thus vitamin D-resistant rickets differs clinically from vitamin D-deficiency rickets both in the hundredfold greater dose required to induce healing and in the fact that a permanent cure is rarely achieved until growth is completed.

Among family members with hypophosphatemia females show considerably less bone disease than males. Very few patients have the



Fig 36-1 Anteroposterior view of the knees of a 4-year-old boy (VI) of 1 kindred of Winters et al [51] showing marked rachitic changes with early healing and lateral curvature of femora and tibiae

muscular weakness and atony that are such prominent and frequent features of vitamin D-deficiency rickets. Only one case of familial vitamin D-resistant rickets with findings suggestive of tetany has been reported [10]. There has been no steatorrhea, liver disease, or renal disease other than the specific abnormalities discussed in the next section. Cranioostenosis and convulsions in infancy have occurred often enough in association with familial vitamin D-resistant rickets to raise the possibility of relationships between them.

The radiologic findings in this syndrome are the same as those seen in rickets or osteomalacia from other causes. In children with rickets the typical changes in the epiphyseal regions of the long bones are the most common and characteristic findings (Figs 36-1, 36-2). Fractures and



Fig 36-2 View of the knees of same patient as that in Fig 36-1 after 3 years of treatment with large doses of vitamin D. Healing is still not complete



Fig 36-3 Anteroposterior view of the humerus of a 58-year-old man (IV-5 of the E kindred of Winters et al [8]) showing coarse trabeculation and a bony protuberance at the site of insertion of the deltoid muscle

pseudofractures and deformities of the skull thorax and long bones are also seen. Additional changes which are occasionally present are similar to those seen in certain osteochondrodystrophies such as coarsened trabecular patterns in long bones, cystic appearing areas in metaphyses and epiphyses, and shortening and broadening of the long bones. Occasionally abnormal bony protuberances appear at the site of major



Fig 36-4 Anteroposterior view of femur of a 41 year-old man (V 10 of E kindred of Winters et al [8]). Note coxa vara, lateral curvature of femur, and pseudofracture of the femoral neck.

muscular attachments and around the joints (Fig 36-3). In adults post-rachitic deformities and rarely pseudofractures occur (Fig 36-4).

Table 36-1 summarizes schematically the principal clinical findings of this disorder. The original reports of familial instances may be consulted for additional details [3: 5-29a].

## HYPOPHOSPHATEMIA AND THE ABNORMALITY IN RENAL EXCRETION OF PHOSPHATE

### HYPOPHOSPHATEMIA

The significant place which a low serum concentration of inorganic phosphate occupies in this disease is highlighted by two lines of evidence

TABLE 36-1 SUMMARY OF FINDINGS IN FAMILIAL HYPOPHOSPHATEMIA AND VITAMIN D RESISTANT RICKETS

| Type of hypophosphatemia  | Age at onset, yr | Clinical and radiologic abnormalities   | Concentration in serum |                     |                         | Other observations   |
|---|------------------|---|------------------------|---------------------|-------------------------|--|
|   |                  |   | Calcium                | Inorganic phosphate | Alkaline phosphatase    |  |
| 1 Asymptomatic hypophosphatemia                                       | Under 1          | Slightly shortened stature  | Normal                 | Low                 | Normal                  | No other abnormalities   |
| 2 Hypophosphatemia in adults with inactive posttraumatic deformities  | Under 1          | Latent (and usually anterior) bowing of legs, shortened stature, occasionally coarsened trabeculation, rarefied areas | Normal                 | Low                 | Normal or slightly high | More frequent anion gap, hypophosphatemic males                                  |
| 3 Hypophosphatemia in adults with deformities and active osteomalacia | Under 1          | Same as for (2) plus pseudo fractures   | Normal                 | Low                 | Slightly high           |  |
| 4 Hypophosphatemia with resistant rickets in child or adult           | Under 1          | Active rickets, occasionally coarsened trabeculation, shortened stature   | Normal or slightly low | Low                 | High                    | More severe in affected males, craniostenosis and convulsions in a few instances |

(1) almost all cases which would be called resistant rickets on other grounds have also had hypophosphatemia (the normal values for serum phosphorus reported by Lernerloth et al [23] in cases with similar clinical features may have been obtained during treatment or those cases may represent a slightly different entity) (2) the probable mode of inheritance has been best revealed by using hypophosphatemia as the discriminant (see below)

In designating a person as hypophosphatemic, due allowance must be made for the normal changes in serum phosphorus with age and for differences between the sexes. This was done by Winters and coworkers [7-9] who expressed mathematically and graphically and with limits of statistical confidence the relationship between serum phosphorus and age for each sex for a large number of observations on normal persons [30]

The age of first appearance of hypophosphatemia has not yet been determined. The present authors have found the detection of hypophosphatemia in a young infant to be difficult even though at a later time the child proves to have the disease. Whether or not this indicates some fundamental age conditioned difference in renal excretion of phosphate or other factors controlling the serum phosphorus in affected individuals is not known.

Among hypophosphatemic individuals in affected families females have slightly higher values for serum phosphorus than males in the same family at all ages.

The alkaline phosphatase level is elevated in affected persons with active rickets or osteomalacia. Treatment with sufficient vitamin D to produce healing of the rickets is accompanied by a return of the alkaline phosphatase to or toward normal. In some instances the serum phosphorus level has risen with treatment but healing has also been observed with no significant rise in phosphate concentration (see Treatment further on).

Other related serum chemical determinations usually give normal results. The serum calcium concentration is normal or rarely slightly low. Serum total protein, albumin and globulin fractions, urea or non protein nitrogen, amino acids, sodium, potassium, chloride, carbon dioxide content and pH are uniformly normal.

In every instance in which it has been studied the hypophosphatemia has been associated with increased renal excretion and decreased renal tubular reabsorption of phosphate. This renal abnormality whatever its origin appears to be the principal cause of the hypophosphatemia. Discussion of this abnormal function requires description of the normal mechanism of phosphate excretion and of factors known to influence it.



## NORMAL MECHANISM FOR RENAL EXCRETION OF PHOSPHATE

The following points have been clearly established

1 At plasma concentrations up to at least 17 mg per 100 ml all the inorganic phosphate of the plasma is ultrafiltrable [31-33]. The phosphate enters the glomerular fluid at virtually the same concentration as that in the plasma [34-35]. Accordingly, the amount of phosphate filtered per unit time is the product of the glomerular filtration rate (as measured by clearance of an agent like inulin) and the plasma concentration of phosphate.

2 In man and dog the net effect of tubular action on glomerular fluid is reabsorption of phosphate: the amount of phosphate excreted is normally only a small fraction of that filtered. The evidence obtained in the dog by Pitts et al. [36] using the stop flow technique suggests that reabsorption of phosphate occurs in the proximal tubule. Only in rare instances [37-40] has evidence for net tubular secretion of inorganic phosphate in man been found. Net tubular secretion of inorganic phosphate has been demonstrated in chickens [41] in cats after infusion of organic phosphates [42] and probably in acidotic dogs [42a].

3 In man and the normal dog a maximal rate of net tubular reabsorption of inorganic phosphate ( $Tm_p$ ) is readily demonstrated. If the concentration of phosphate in the plasma is raised progressively the calculated rate of net reabsorption of phosphate (phosphate filtered minus phosphate excreted) reaches a constant value and further increments in filtered phosphate are accompanied by a corresponding increase in excreted phosphate. When measured under standardized conditions the values for  $Tm_p$  are reasonably constant in normal individuals on normal diets. Most reported normal values for  $Tm_p$  for children and adults are between 120 and 150  $\mu$ m phosphate reabsorbed per 100 ml glomerular filtrate although a few supposedly normal individuals have had values as low as 80  $\mu$ m per 100 ml glomerular filtrate [43-49]. These results do not exclude tubular secretion of phosphate but they do indicate that if secretion occurs it must be at a constant rate and must be unaffected by progressive rises in plasma phosphate concentration.

The  $Tm_p$  is the most meaningful physiologic measurement of renal tubular activity in any consideration of phosphate excretion. Other appraisals of the renal mechanism for excretion of phosphate have frequently been used. Among these are the renal clearance of phosphate ( $C_{PO}$  = excreted phosphate/serum phosphorus) the percentage of filtered phosphate that is reabsorbed (%TRP) and the fraction of filtered phosphate that is not reabsorbed (=  $C_{PO}/C_I$ ). These estimations are made without loading the excretory mechanism with phosphate. Since these three calculations are all influenced by the serum phosphate concentration as well as by glomerular and tubular function direct interpretation of

tubular activity from them is extremely difficult. Their use has at times led to confusing and erroneous views. In patient IV 5 of Table 36-3 for example before phosphate loading the  $C_{\text{Po}}$  was 13 ml per min and the %TRP was 87 both within the range of 'normal' yet the  $Tm_{\text{P}}$  was distinctly low. (This patient had normal intake of phosphate prior to the  $\Pi$  measurements; an unusually high or low intake will be reflected in changes in  $C_{\text{Po}}$ , %TRP and  $C_{\text{Po}}/C_{\text{I}}$  because of the effect on serum phosphate concentration without any change necessarily in tubular function.) As suggested by Lambert et al [46] and Milne et al [50] care should be taken to compare these calculated values with those obtained for normal subjects at the same level of serum phosphorus. When the glomerular filtration rate is normal (see below) it may be possible thus to separate abnormal from normal phosphate excretory patterns. In the example cited above a  $C_{\text{Po}}$  of 13 ml per min is actually probably high for a serum phosphorus of 2.4 mg per 100 ml.

In the presence of renal disease with significant reduction in glomerular filtration rate the (unloaded) tubular reabsorption of phosphate is usually depressed out of proportion to the decrease in glomerular filtration [51]. It is clear that interpretations of tubular reabsorption of phosphate in other abnormal states cannot be made when there is significant concomitant reduction of glomerular filtration. This change in tubular reabsorption may conceivably be related to increased parathyroid function (see Hormonal Factors below).

#### FACTORS KNOWN OR THOUGHT TO INFLUENCE THE NORMAL MECHANISM FOR EXCRETION OF PHOSPHATE

##### *Possible Effects of Acute Changes in Glomerular Filtration Rate*

In the dog changing the glomerular filtration rate—by varying the protein intake—causes no change in  $Tm_{\text{P}}$  [52]. Evidence in man is less clear-cut partly because of the difficulty of producing large changes in glomerular filtration rate [45–47]. Gross abnormalities of tubular reabsorption as seen for example in hyperparathyroidism and familial hypophosphatemia may occur without any evidence of changes in glomerular filtration rate [8, 49–53].

##### *Hormonal Factors*

Intramuscular administration of extracts of bovine parathyroid glands over several days to normal subjects and hypoparathyroid patients produces an increased excretion of phosphate, a fall in serum phosphorus and a fall in  $Tm_{\text{P}}$  [54]. Patients with proved hyperparathyroidism have low values for  $Tm_{\text{P}}$  and the  $Tm_{\text{P}}$  eventually returns to normal or nearly normal after removal of the overactive parathyroid tissue [49–53, 55], provided there has been no permanent renal damage. Thus there is little

## NORMAL MECHANISM FOR RENAL EXCRETION OF PHOSPHATE

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on tubular reabsorption of phosphate are needed. The evidence presented to date is that parathyroid hormone (which has many actions similar to those of the vitamin D group of sterols), cortisone, possibly stilbestrol, and possibly vitamin D under certain circumstances (see below) all decrease the tubular reabsorption of phosphate. This suggests a common mechanism of action on the renal tubules. The possibility that abnormal steroids may be involved in vitamin D-resistant rickets is discussed further on under Possible Mechanisms of Pathogenesis. Other relationships between cortisone and vitamin D are discussed under Other Possible Mechanisms of Pathogenesis.

### *Chemical Substances Possibly Competing for Tubular Reabsorption of Phosphate*

A number of substances appear to compete with inorganic phosphate for renal tubular reabsorption. Thus, when the filtered load of any of these is increased and its rate of tubular reabsorption increases, the tubular maximum for reabsorption of phosphate is simultaneously depressed. Glucose [72-73], acetoacetate [74], bicarbonate [75], glycine, and alanine [52] are known to have this effect. It seems probable that glucose and acetoacetate affect phosphate reabsorption through a mechanism different from that of amino acids and bicarbonate. No direct studies of the simultaneous effects of these agents have been made. Chlorzine, in doses which inhibit or block tubular reabsorption of glucose, increases the tubular reabsorption of phosphate. This suggests that even the normal rate of reabsorption of glucose has a slightly suppressive effect on phosphate reabsorption [72-73]. The carbonic anhydrase inhibitor acetazolamide depresses the  $Tm_p$  concomitant with increasing bicarbonate excretion [75-76].

The interrelationships between the pathways for tubular reabsorption of these substances are quite complex and in need of much further study. Lotpeich discusses this problem in relation to the hypothetical biochemical pathway which actively transfers phosphate across the tubular cell membrane [76a]. The inference that the inhibition may be of a competitive type is pertinent to the decreased tubular reabsorption of phosphate in vitamin D-resistant rickets. The occasional appearance of slight glucosuria and aminoaciduria in this disorder is compatible with possible abnormalities in the metabolism of these agents in the production of the phosphaturia. These interrelationships are discussed in more detail in Chap. 37.

There is equivocal evidence concerning the effects of hypokalemia on the tubular reabsorption of phosphate. Hogben and Bollman [32] found that potassium infusion produced a rise in  $Tm_p$ , but the effect was inconsistent. In one patient with potassium deficiency and disease, Mahler and Stranbury [77] found increased excretion of phosphate despite a low

doubt that one specific action of the parathyroid hormone is on renal tubular reabsorption of phosphate. Earlier studies of this action are referred to by Bartter [44] and Lotspeich [56].

The effects of varying intakes of calcium and phosphate on renal tubular reabsorption of phosphate are probably mediated through the parathyroid glands. Prolonged increased intake of phosphate results in a reduction in the amount of phosphate reabsorbed by the renal tubule [57]. Prolonged elevation of serum phosphorus level by repeated infusions leads to a marked fall in  $Tm_p$  in normal persons but probably not in hypoparathyroid patients [58]. A low intake of calcium produces parathyroid hyperplasia in animals [59-60]. Secondary hyperplasia of the parathyroid glands in patients with renal disease is associated either with decreased total calcium concentration in the serum [61] (and decreased ionized calcium of the serum [62]) or with elevated serum phosphorus concentration or both. Calcium infusion is usually followed by a temporary increase in tubular reabsorption of phosphate (unloaded) and in  $Tm_p$  [63-66]. All these findings are best interpreted as indicating that a low calcium intake or absorption or a high phosphorus intake leads to parathyroid hyperplasia which in turn leads to decreased tubular reabsorption of phosphate.

There is evidence that in the chicken parathyroid hormone increases the net tubular secretion of phosphate [41]. Since tubular secretion of phosphate in man has not been excluded the possibility must be kept in mind that parathyroid hormone also affects tubular secretion in man.

Certain other hormones or related compounds have been studied for their effects on renal phosphate excretion. Cortisone given intravenously to dogs produces an immediate fall in  $Tm_p$  but deoxycorticosterone does not [67-68]. The evidence is less clear cut in man. ACTH and cortisone given in large amounts for several days cause increased phosphate excretion because of a decrease in the net amount of phosphate reabsorbed [40]. In one experiment the evidence suggested net tubular secretion. In acute studies using intravenous hydrocortisone no change in  $Tm_p$  in some normal subjects and a slightly decreased  $Tm_p$  in others was found [69]. A decrease was more frequent when  $Tm_p$  was expressed per unit volume of glomerular filtrate [69]. Diethylstilbestrol given for 14 to 21 days to five patients (three with osteoporosis) was followed by some decrease in  $Tm_p$  in each [70]. One cannot say from these data whether stilbestrol has a direct action on the renal tubules.

Triiodothyronine given intravenously to dogs in 1 mg doses induced increased renal excretion of phosphate and it was stated that tubular reabsorption was decreased [71]. However serum phosphorus level and glomerular filtration rate rose and in the published data the calculated rate of tubular reabsorption actually increased.

Further studies of the action of these hormones and related compounds

compared with the  $Tm_p$  of a previous day. The data are included in Table 36-4. Glomerular filtration rate did not change. Huge doses of vitamin D given to normal dogs (1.25 to 3 mg per kg given over 5 days) depress glomerular filtration rate as well as  $Tm_p$ , but the fall in  $Tm_p$  is out of proportion to the decline in glomerular filtration rate [88].

These results may represent an action of massive doses of vitamin D and related compounds which is shared with other sterols and which is different from the physiologic effects of vitamin D on the kidney.

Vitamin D in large doses raises the serum concentration and excretion of citrate to abnormally high levels [89-90]. Since infusing citrate into a hypoparathyroid subject does not produce increased phosphate excretion [80] whereas vitamin D has this effect in the same type of subject [84-86], it seems unlikely that vitamin D affects phosphate excretion through its effect on citrate metabolism. More definitive studies of this point are needed.

#### THE RENAL ABNORMALITY IN PHOSPHATE EXCRETION

Beginning with the observations of Robertson Harris and McCune [2] in 1942 many studies of patients with vitamin D resistant rickets

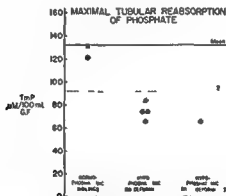


Fig. 36-5 Values for maximal tubular reabsorption of phosphate ( $Tm_p$   $\mu$  moles per 100 ml glomerular filtrate) on 10 members of the Hurler kindred of Winters et al. ■ = male, ● = female. The mean and two standard deviations (—) for the values of  $Tm_p$  of 14 normal subjects [3, 5, 45 and unpublished observations of the authors] are represented by the lines.

have shown abnormally high rates of excretion of phosphate or clearances of phosphate, especially when considered with respect to their low values of serum phosphorus [3, 5, 17, 21, 23, 29, 37, 91-93]. This has been true both in cases with a clear-cut familial incidence [3, 23, 29, 37] and in other cases lacking an obvious family history.

Unequivocal demonstration of the nature of the principal renal abnormality in this disease though not of its mechanism has come from

serum phosphorus level when the patient was hypokalemic. Phosphate excretion and serum phosphorus level returned to normal when the potassium deficiency was corrected but other changes were going on simultaneously, such as the correction of a metabolic acidosis. As noted above, deoxycorticosterone had no effect on  $Tm_p$  in an experiment in which serum potassium concentration fell from 3.8 to 2.6 mEq per l [67].

Systemic acidosis is accompanied by decreased tubular reabsorption of phosphate in the nonloaded state in the human being [78], in the dog conflicting evidence has been presented [72-79].  $Tm_p$  is not affected by acidosis in either species.

Nordin and Fraser [80] found that infusion of sodium citrate led to increased phosphate excretion without significant change in serum phosphate concentration. Glomerular filtration rate was not measured but if it did not change, then tubular reabsorption of phosphate was diminished. This response did not occur in one hypoparathyroid subject and Nordin and Fraser suggested that the effect of citrate was mediated through the parathyroid gland, i.e., that the infused citrate formed a complex with the ionized calcium of serum, thereby lowering ionized calcium and stimulating parathyroid hormone secretion. The rate of citrate excretion was not measured. The direct role of citrate excretion if any, in phosphate excretion has not been evaluated.

#### *Action of Vitamin D on Renal Tubular Function and Excretion of Phosphate*

Existing information does not permit an unequivocal statement concerning the effects of vitamin D on renal tubular function in normal or diseased states.

Vitamin D given to vitamin D-deficient rachitic patients and dogs produces an increased tubular reabsorption of phosphate [81] and increased  $Tm_p$  [82, 83]. This result could be a direct effect of vitamin D on the renal tubule. It might also be an indirect effect, with the well established action of vitamin D in increasing calcium absorption resulting in suppression of parathyroid function. As Albright has pointed out [81] these two possibilities could be differentiated by repeating the study on rachitic dogs with and without parathyroidectomy, but this has not yet been done.

In hypoparathyroid human subjects vitamin D in massive doses (equivalent to 5 to 10 mg calciferol a day—1 000 times the maintenance requirement of vitamin D) and dihydrotachysterol (A.T. 10) 6 to 12 mg per day have both produced significant increases in phosphate excretion concomitant with a falling serum phosphorus [84-86]. Unless glomerular filtration rate changed these results indicate decreased tubular reabsorption of phosphate.

Rupp and Swoboda [87] gave 20 mg of a vitamin D<sub>2</sub>-hydroxol intravenously to one normal subject and found a slightly lower  $Tm_p$  when

| Subject's name | Sex | Height<br>in | Values before phosphate load |            |                                       | Values after phosphate load | Rate of phosphate excretion<br>$\mu\text{M}/100\text{ ml GF}$ |
|----------------|-----|--------------|------------------------------|------------|---------------------------------------|-----------------------------|---|
|                |     |              | Protein                      | Creatinine | Excretion<br>$\mu\text{M}/\text{min}$ |                             |   |
|                |     |              |                              |            |                                       |                             |   |

## A Hypophosphatemic patients with deformities

|       |    |   |    |      |      |     |      |      |     |     |
|-------|----|---|----|------|------|-----|------|------|-----|-----|
| V 1   | 41 | M | 65 | 1.8  | 0.41 | 144 | 13.8 | 31.6 | 135 | 0.7 |
| V 11  |    |   |    | 1.36 | 0.63 | 131 | 18.5 | 60.3 | 170 | 0.1 |
| V 3   | 58 | M | 68 | 2.45 | 0.80 | 98  | 10.6 | 51.2 | 101 | 5.4 |
| V 51  |    |   |    | 2.11 | 0.68 |     | 3.0  |      | 111 | 5.6 |
| V 51  |    |   |    | 2.40 | 0.7  | 105 | 20.0 | 58.0 | 93  | 8.1 |
| V 133 | 3  | M | 67 | 1.8  | 0.58 |     |      |      | 166 | 7.3 |
| V 13  | 8  | F | 67 | 2.70 | 0.41 |     |      |      | 33  | 6.8 |

## B Hypophosphatemic patients with no deformities

|       |    |   |    |      |      |     |     |      |     |     |
|-------|----|---|----|------|------|-----|-----|------|-----|-----|
| V 3   | 31 | F | 64 | 2.48 | 0.80 | 113 | 0.9 | 31.7 | 130 | 0.1 |
| V 4   |    | F | 6  | 2.14 | 0.69 |     |     |      | 103 | 1.5 |
| V 103 | 3  | F | 64 | 2.00 | 0.81 |     |     |      | 92  | 6.6 |
| V 8   |    | F | 6  | 2.40 | 0.78 |     |     |      | 134 | 1.5 |

## C Normal healthy subjects

|     |    |   |    |      |      |  |  |  |      |      |
|-----|----|---|----|------|------|--|--|--|------|------|
| V 8 |    | F | 68 | 3.00 | 0.41 |  |  |  | 1.3  | 1.01 |
| V 5 | 31 | M | 71 | 3.54 | 1.14 |  |  |  | 1.41 | 1.31 |

Column headings:  $V$  = serum concentration of inorganic phosphorus;  $C_r$  = clearance of inulin;  $L$  or  $I$  = rate of phosphate excretion;  $H$  = rate of phosphate reabsorption;  $GFR$  = glomerular filtrate.

† After vitamin D 300,000 IU/day for 6 months.

‡ After low-calcium diet plus Basaljel 180 ml/day for 7 days.

§ Normal diet intravenous loading with calcium gluconate (13 mg calcium per kg over 6 hr). Value of rephosphate loading were obtained at 4 to 5 hr after phosphate loading at 5 to 6 hr after start of calcium infusion.

Source: H. W. Winters et al. [5] and previously unpublished observations of the authors.



TABLE 36-2 SUMMARY OF EVIDENCE ON PHOSPHATE EXCRETION IN PATIENTS WITH VITAMIN D-DEFICIENT RICKETS RECALCULATED

| Adult and/or Child (yr) | Age at onset (yr) | Sex | GFR ml/min          | Urine P (mg/100 ml) | Cr (mg/dl) | Cr (ml/min) | % absorbed (calculated from P balance) | % absorbed (calculated from P balance) | Final P (mg/100 ml) |
|-------------------------|-------------------|-----|---------------------|---------------------|------------|-------------|--|--|---------------------|
| Adult (19)              | 18                | F   | Blood 100 mg/100 ml |                     |            |             |  |  |                     |
| Child (19)              | 13                | F   | NPN 26 mg/100 ml    |                     |            |             |  |  |                     |
| Adult (19)              | 14                | F   | NPN 9 mg/100 ml     |                     |            |             |  |  |                     |
| Child (19)              | 14                | F   | Cr 99               |                     |            |             |  |  |                     |
| Adult (19)              | 15                | F   | BLN 14 g/100 ml     |                     |            |             |  |  |                     |
| Child (19)              | 15                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 16                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 16                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 17                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 17                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 18                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 18                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 19                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 19                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 20                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 20                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 21                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 21                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 22                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 22                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 23                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 23                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 24                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 24                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 25                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 25                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 26                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 26                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 27                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 27                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 28                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 28                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 29                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 29                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 30                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 30                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 31                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 31                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 32                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 32                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 33                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 33                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 34                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 34                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 35                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 35                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 36                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 36                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 37                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 37                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 38                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 38                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 39                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 39                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 40                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 40                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 41                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 41                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 42                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 42                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 43                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 43                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 44                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 44                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 45                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 45                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 46                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 46                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 47                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 47                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 48                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 48                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 49                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 49                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 50                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 50                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 51                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 51                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 52                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 52                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 53                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 53                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 54                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 54                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 55                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 55                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 56                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 56                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 57                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 57                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 58                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 58                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 59                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 59                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 60                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 60                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 61                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 61                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 62                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 62                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 63                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 63                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 64                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 64                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 65                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 65                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 66                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 66                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 67                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 67                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 68                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 68                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 69                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 69                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 70                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 70                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 71                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 71                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 72                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 72                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 73                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 73                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 74                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 74                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 75                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 75                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 76                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 76                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 77                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 77                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 78                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 78                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 79                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 79                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 80                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 80                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 81                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 81                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 82                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 82                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 83                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 83                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 84                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 84                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 85                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 85                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 86                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 86                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 87                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 87                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 88                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 88                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 89                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 89                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 90                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 90                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 91                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 91                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 92                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 92                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 93                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 93                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 94                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 94                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 95                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 95                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 96                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 96                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 97                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 97                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 98                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 98                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 99                | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 99                | F   |                     |                     |            |             |  |  |                     |
| Adult (19)              | 100               | F   |                     |                     |            |             |  |  |                     |
| Child (19)              | 100               | F   |                     |                     |            |             |  |  |                     |

arginine [97] or methionine [99] are probably abnormal in patients with vitamin D-resistant rickets but more specific information is needed on the disease of the subjects and on comparable results in normal persons.

There is meager evidence for an association of renal glycosuria with vitamin D-resistant rickets. The only patients described so far with this combination and with family histories of vitamin D-resistant rickets are the young boy reported by Fanconi and Girardet [17] and Tobler et al [29] who was unusual in several other ways and one of two brothers both of whom were found by Dent [91] to have vitamin D-resistant rickets. Only rare instances of glycosuria have been reported among nonfamilial cases [100-101]. The large body of negative evidence for renal glycosuria in patients with familial hypophosphatemia and vitamin D-resistant rickets suggests that an abnormality in glucose reabsorption does not participate in the production of the increased phosphate excretion. Quantitative determinations of tubular maxima for glucose reabsorption in subjects with clear family histories are needed to clarify this point.

The glomerular filtration rate has been normal in the patients in whom it has been measured (Tables 36-2, 36-3). Freeman and Dunskey [100] also found a normal exogenous creatinine clearance in their patient. Acidification of the urine is probably normal as shown by normal  $\text{CO}_2$  content of the blood. The only exceptions have been isolated low values for  $\text{CO}_2$  content in the child reported by Fanconi and Girardet [17] and in one patient reported by Snoboda [28]. In addition a few observers have found a normal response to an ammonium chloride load [21, 94, 102]. Concentrating ability is normal as are concentration and dilution tests [17, 21]. There is no evidence of abnormalities in sodium or potassium excretion and serum concentrations are normal. Renal response to administration of parathyroid hormone is probably normal (see below). Response to other agents like cortisone has not been determined. There are no reports of more quantitative measures of renal function such as the tubular maxima for excretion of para aminohippurate.

The renal abnormality in familial hypophosphatemia thus appears to involve solely the tubular reabsorption of phosphate. More quantitative information is needed in certain areas: in particular the response of subjects with clearly defined genetic backgrounds to loading with amino acids, glucose and para aminohippurate. An equally great need is for histologic and histochemical study of renal tissue. Such information is entirely lacking.

#### EFFECTS OF VITAMIN D ON RENAL FUNCTION IN VITAMIN D-RESISTANT RICKETS

The renal effects of vitamin D in this disorder have been incompletely studied. The administration of large doses of vitamin D causes either no

measurement of  $Tm_F$  by Rupp and Swoboda [87] and by Winters et al [7, 8]. In both studies the  $Tm_F$  was distinctly low. Tables 36.2 and 36.3 and Fig. 36.5 present the available data. The values for  $Tm_F$  in members of the "C" kindred of Winters et al. were about equally low in four adults with hypophosphatemia without bone disease and in four adults with hypophosphatemia and bone disease.

The hypophosphatemia appears to be almost entirely due to decreased tubular reabsorption of phosphate. Phosphate balance may be otherwise normal (see below). At sufficiently low serum phosphate levels such as when intake is very low, phosphate balance is maintained with minimal phosphate excretion [94].

The possibility that extrarenal factors such as parathyroid hormone are involved in the pathogenesis of the decreased renal tubular reabsorption of phosphate is discussed below, under Possible Mechanisms of Pathogenesis.

#### OTHER RENAL ABNORMALITIES

Except for the low tubular reabsorption of phosphate, renal function is normal as far as can be determined from present data. The occurrence of slight degrees of aminoaciduria and glycosuria in a few cases suggests that in some instances of this disease there may be additional defects similar to those in Fanconi syndrome. In attempting to evaluate the degree of association if any between familial hypophosphatemia and the Fanconi syndrome, the study of families with well documented genetic patterns should prove particularly valuable. In one of the families most carefully studied from a genetic viewpoint, total amino acid excretion in five hypophosphatemic persons was normal [8]. Paper chromatograms of the amino acids of the urine of a number of affected adults and children in the families studied by these authors also gave normal results [8a]. All other determinations of aminoaciduria in familial instances of the disorder have been normal [18, 21, 23, 29] except for one case first reported by Fanconi and Girardet [17]. This child had slightly elevated serum amino acid concentration on one occasion and slightly increased urinary excretion of amino acids on three occasions. The patient also had slight glycosuria [29], this coupled with unusual facies and an abnormal skull x-ray led Tobler and coworkers to question whether he had more than simple vitamin D-resistant rickets [29].

Observations on aminoaciduria in patients with vitamin D-resistant rickets without family history have been more varied. Some patients have had no abnormal aminoaciduria [25, 29, 96]. In others abnormally high excretion of various amino acids has decreased upon administration of large doses of vitamin D [21, 25, 97], a response similar to that of the aminoaciduria of vitamin D-deficiency rickets to smaller doses [97, 98]. The excretory patterns of amino acids after loading with histidine-

excretion of these substances. This response was recorded throughout two to six 6-day collection periods. Since there were no measurements of glomerular filtration rate it is not known whether any change in tubular reabsorption of phosphate occurred. If glomerular filtration rate did not change significantly then tubular reabsorption of phosphate must have decreased. The authors proposed that this indeed did happen and may have been due to stimulation of parathyroid hormone by the fall in serum calcium concentration.

TABLE 36-4 ACUTE EFFECT OF INTRAVENOUS VITAMIN D ON MAXIMAL TUBULAR REABSORPTION OF PHOSPHATE ( $T_{mp}$ )

| Case No | Dose of vitamin D | Range of $T_{mp}$ |                             |                        |
|---------|-------------------|-------------------|-----------------------------|------------------------|
|         |                   | Before vitamin D  | Immediately after vitamin D | 1 week after vitamin D |
| 5 (RR)† | 0 mg              | 97-100            | 91-101                      | 103-119                |
| 5 (RR)  | 10 mg             | 100-145‡          | 144-147                     |                        |
| 3 (RR)  | 10 mg × 2         | 69-84‡            | 110-113                     | 5-8                    |
| Normal  | 10 mg × 2         | 174-208‡          | 143-148                     | 172-175                |
| 1 (RR)  |                   | 65-100            |                             | 4-172                  |

Expressed in micromoles per 100 ml glomerular filtrate

† RR = resistant rickets

‡ The 3 control measurements of  $T_{mp}$  were done on a previous day and do not represent control values immediately prior to injection of vitamin D

§ Not recalculated from Rupp and Swoboda [8]

In summary, large doses of vitamin D may result in increased renal tubular reabsorption of phosphate in patients with familial hypophosphatemia and vitamin D-resistant rickets. Even if true, this is not necessarily a direct action of vitamin D. The proposal originally made by Albright et al [84, 86] and subsequently by numerous students of this disease—that the purported change in tubular reabsorption is the result of diminished parathyroid activity as calcium absorption improves—has yet to be proved or disproved.

## GASTROINTESTINAL ABSORPTION OF CALCIUM AND PHOSPHATE

### NORMAL FEATURES AND THE ROLE OF VITAMIN D

Normal adults and children supplied with physiologic amounts of vitamin D and dietary calcium above a minimal level (0.45 to 1.0 gm per day [107, 108]) absorb enough calcium to remain in calcium balance. Children achieve enough positive balance for normal growth of bone. A deficiency of vitamin D intake uniformly leads to progressively lowered absorption of calcium until fecal excretion of calcium equals intake.

change or a rise in serum phosphorus level accompanied by either no change or a fall in urinary phosphate excretion [1, 2, 17, 94, 102]. Whether these changes are the result of decreased glomerular filtration rate or increased renal tubular reabsorption of phosphate is not known and cannot be determined from measurement of phosphate clearance alone. The studies reported by Robinson and Nelson [103] in which  $C_{Cr}$  remained constant as serum phosphorus concentration rose with vitamin D therapy, offer an illustration of the care with which clearance data must be interpreted. If glomerular filtration rate remained constant as inferred in four of six subjects a constant  $C_{Cr}$  in association with a rising serum phosphorus concentration requires a simultaneous increase in both phosphorus reabsorption and excretion in these subjects. Only if there were a considerable fall in glomerular filtration rate could the results of Robinson and Nelson indicate no change or a decrease in tubular reabsorption of phosphate with vitamin D therapy.

There have been few direct measurements of glomerular filtration rate in relation to vitamin D therapy. In one subject stated to have familial vitamin D-resistant rickets Klein and Gow [104] found a rise in inulin clearance one day after giving 600 000 units (22 mg) of vitamin D intramuscularly. Robinson and Nelson [103] described a fall in glomerular filtration rate in two of six subjects with vitamin D-resistant rickets on prolonged vitamin D therapy. In one subject with this disease reported by Dent and Harris [5] daily creatinine excretion did not change during vitamin D administration.

Rupp and Swoboda [87] have reported the acute response of phosphate excretion to intravenous vitamin D administration both in nonloaded and phosphate-loaded patients. They studied three children with familial vitamin D-resistant rickets. Glomerular filtration rate was little affected. The effects on  $Tm_p$  are summarized in Table 36-4. In two patients  $Tm_p$  was higher immediately after administration of vitamin D and in one it did not change. There was no change in phosphate reabsorption in one subject studied without phosphate loading. These data cannot be considered conclusive evidence for a direct effect of vitamin D on phosphate reabsorption.

Measurements of  $Tm_p$  and phosphate reabsorption in the nonloaded state 1 week after these acute intravenous doses were given were not different from control observations [87]. If vitamin D has an effect on renal function it is short lived. In a preliminary report Dent has stated that there is a rise in  $Tm_p$  with administration of vitamin D [105]. The route, amount and duration were not specified.

Zetterstrom et al [106] made the curious and thus far unique observation in two children with vitamin D-resistant rickets that the initial response to 100 000 to 200 000 I U per day orally was a fall in serum concentrations of phosphate and calcium without change in absorption or

in vitamin D-resistant rickets is furnished by the studies of Saville et al [114] and Fraser et al [119]. These workers found that administration of large amounts of phosphate either orally or intravenously without large doses of vitamin D will induce increased positive balances of calcium and phosphorus and initiate healing of the rickets. Studies of the absorption of calcium and phosphate in affected members of families with the best worked out pedigrees based on hypophosphatemia have not been made.

In summary the gastrointestinal absorption of calcium and secondarily of phosphate is probably abnormally low. This is particularly evident in children with active rickets who do not have a sufficiently positive balance for normal growth. In affected family members with asymptomatic hypophosphatemia impairment in calcium and phosphate absorption may not be apparent. Bony development is normal both clinically and radiologically. Vitamin D in massive doses will induce increased absorption of calcium and phosphorus. This is probably no different from what occurs in normal persons despite the lack of response to physiologic amounts of vitamin D. Large increases in intake of phosphate also produce increased absorption of phosphate and probably of calcium.

### ABNORMALITIES IN METABOLISM OF BONE

A thorough discussion of the normal mechanism of formation of bone and the pathogenesis of rickets due to deficiency of vitamin D is beyond the scope of this chapter; good descriptions may be found in Eliot and Park [120], Neuman and Neuman [121] and Bourne [122].

In considering the bony abnormalities found in familial hypophosphatemia and vitamin D-resistant rickets it must be remembered that this inherited disorder can be manifested by hypophosphatemia and diminished tubular reabsorption of phosphate alone with *no apparent bone disease*. Although no bone tissue has been examined histologically from such subjects there are no radiologic abnormalities. The only clinically discernible abnormality is slightly shorter stature than that of normophosphatemic siblings [8]. This virtual lack of bone disease despite hypophosphatemia indicates (1) that the genetically transmitted abnormality is not concerned directly with bone metabolism and (2) that factors other than simple hypophosphatemia are participating in the disease in patients who develop bone disease.

Two different types of bony abnormalities have been described in patients with vitamin D-resistant rickets. The most characteristic finding and the one which gives the disease its name is the typically rachitic appearance of the epiphyseal region. There is a markedly expanded zone of proliferating cartilage with increased osteoid tissue and invasion by wide tortuous blood vessels [1, 16, 28, 94, 123, 124]. The e

Urinary calcium excretion may become virtually zero. Studies of animals and man on calcium free diets show that little if any of the fecal calcium is due to calcium excretion into the gastrointestinal tract [84, 86, 110, 111]. The administration of vitamin D to vitamin D-deficient subjects leads to an increase in calcium absorption [111-113]. This action of vitamin D is the most clearly established role of the vitamin in maintaining normal metabolism of calcium and phosphate. The mechanism of action has not been determined.

Intestinal absorption of phosphate is probably not directly dependent upon vitamin D [110]. In vitamin D-deficient animals or man receiving calcium the phosphate absorption is low. This is thought to be secondary to poor absorption of calcium and the consequent sequestration of calcium phosphate within the intestine [84, 110].

#### ABNORMALITIES IN ABSORPTION OF CALCIUM AND PHOSPHORUS IN FAMILIAL HYPOPHOSPHATEMIA AND VITAMIN D-RESISTANT RICKETS

Children with vitamin D-resistant rickets before treatment with massive doses of vitamin D excrete virtually all their ingested calcium in the feces. Urinary calcium excretion is low. These subjects have slightly positive or slightly negative balances for calcium but they clearly lack the positive balance which is characteristic of the normal growing child. Total phosphate balance is comparable to the calcium balance but a larger proportion of the ingested phosphate is absorbed and excreted in the urine. The findings are similar in the few studies of patients with vitamin D-resistant rickets with [3, 5, 17, 114] and without family histories [1, 94, 115, 116].

In vitamin D-resistant rickets large doses of vitamin D (2.5 to 37.5 mg per day) uniformly increase the absorption of calcium while urinary excretion remains low. Simultaneously the phosphate balance becomes positive primarily because of increased absorption. This response to very large doses of vitamin D is similar to the response of patients with vitamin D-deficiency rickets to smaller doses. Dihydrotachysterol (A.T. 10) in doses in the same range as those of vitamin D (1.25 to 12.5 mg per day) produces similar changes in calcium absorption in vitamin D-resistant rickets [86, 114, 117].

The response of patients with resistant rickets to massive doses of vitamin D is similar to that of other subjects. Albright and his coworkers [84-86] for example found comparable increases in calcium absorption in patients with primary hypoparathyroidism and patients with idiopathic hypercalcaemia given large doses of vitamin D or dihydrotachysterol. Normal man and normal rats also have increased calcium absorption when given large doses of vitamin D [111, 118].

Further evidence that poor absorption of calcium is not the only defect

in some areas. Toxic doses of vitamin D clearly produce abnormal amounts of bone resorption [111]. Bauer et al [11] in studies in man found that the estimated increase in total daily accretion of phosphate salt into bone due to vitamin D was two to six times the extra retention of phosphate from the gastrointestinal tract promoted by vitamin D. The changes were of similar magnitude in two subjects with vitamin D-deficiency rickets treated with 30 000 to 45 000 I U per day and in two subjects with vitamin D-resistant rickets treated with 150 000 to 500 000 I U per day. Thus it appears that under the influence of vitamin D calcium and phosphate are mobilized from one part of bone to be deposited in another.

As Nicolaysen and Feg Iarsen have pointed out [111] it is both important and difficult in attempting to establish the actions of vitamin D to distinguish between effects of vitamin D on calcium absorption and direct effects on bone. The same problem exists in the study of the pathologic physiology of vitamin D-resistant rickets. One cannot say from available evidence whether the response of the bone to normal levels of vitamin D would be normal if the serum concentrations of calcium and phosphate were maintained at normal levels for prolonged periods by means other than very large doses of vitamin D.

The similarity of the changes in metaphysis and diaphysis to those seen in osteochondrodystrophy raises the possibility that this bony abnormality is another congenital presumably inherited defect in these patients and not directly related to hypophosphatemia or to vitamin D. The lesion could also be a direct effect of parathyroid overactivity although the changes described have not been entirely typical.

### POSSIBLE MECHANISMS OF PATHOGENESIS AND QUESTIONS STILL UNANSWERED

This discussion of pathogenesis of familial hypophosphatemia and vitamin D-resistant rickets will be focused on the two abnormalities which the authors consider to be the most characteristic features of the disorder: hypophosphatemia and decreased renal tubular reabsorption of phosphate. The other abnormalities thus far identified—low gastrointestinal absorption of calcium and the bony abnormalities—will be discussed in so far as they bear upon the pathogenetic possibilities to be presented. A final decision about mechanism cannot now be made.

#### *A Genetically Determined Intrinsic Renal Tubular Defect in Reabsorption of Phosphate*

This possibility offers the simplest explanation for the occurrence of hypophosphatemia and decreased renal tubular reabsorption of phosphate. Consistent with this hypothesis are the facts that hypopho-



changes are present in cases with and without family histories. The radiologic changes in the epiphyseal area are entirely characteristic of vitamin D-deficiency rickets.

A second type of abnormality has been found in the compact bone of the metaphyses of long bones. In biopsies from such areas in two cases Engfeldt et al. [16] observed an abnormal, irregular mosaic formation of the Haversian system and trabeculae, very little osteoid and probably an increase in osteoblastic borders and "resorption cavities." Others have described similar changes [3, 29]. These abnormalities may be present before treatment with massive doses of vitamin D as well as afterwards [16]. The histologic pattern has some features suggestive of Paget's disease or of experimental hyperparathyroidism [125]. These changes are not always present. In some patients bone from metaphysis has been almost normal [17, 29].

The epiphyseal lesion typical of rickets heals in most instances when the patients are treated with massive doses of vitamin D. However the healing of the epiphyseal lesion is apparently not dependent upon an action of vitamin D on the bone itself but rather upon its action in promoting absorption of calcium and phosphate. Healing can be initiated by supplying large amounts of phosphate intravenously or orally [119] and is accelerated by immobilization of patients at the time of osteotomy without large doses of the vitamin [21, 26, 29, 102]. Vitamin D-resistant rickets and vitamin D-deficiency rickets are alike in not requiring vitamin D for the healing of the epiphyseal lesion. Healing is also initiated in vitamin D-deficiency rickets by supplying large amounts of phosphate intravenously [119] or citric acid-sodium citrate orally [126-128]. An unexplained observation which may be a valuable clue to the difference between vitamin D-deficiency rickets and vitamin D-resistant rickets is that the latter does not heal with administration of citric acid-sodium citrate [12, 23, 102, 120, 129]. Further study of this difference in response to citrate is needed.

The second type of abnormality of bone structure in vitamin D-resistant rickets is more difficult to interpret. This distortion of trabecular structure may simply be another manifestation of rickets slow to develop and slow to heal and accordingly more evident in vitamin D-resistant rickets than in vitamin D-deficiency rickets. In rats [111, 130] and dogs [131] an abnormal trabecular appearance and increase in noncalcium content of the shaft of long bones is a distinctive feature of vitamin D deficiency and is not directly related to absorption of calcium and phosphate. This has suggested to Nicolaysen and coworkers [111, 130] and to Mellanby [131] that vitamin D has a direct function in normal bone formation. Furthermore Carlsson [132] and Nicolaysen and Eeg Larsen [133] have presented evidence in vitamin D-deficient rats which suggests that vitamin D in physiologic doses causes increased bone resorption.

in some areas. Toxic doses of vitamin D clearly produce abnormal amounts of bone resorption [111]. Bauer et al [11] in studies in man found that the estimated increase in total daily accretion of phosphate salt into bone due to vitamin D was two to six times the extra retention of phosphate from the gastrointestinal tract promoted by vitamin D. The changes were of similar magnitude in two subjects with vitamin D-deficiency rickets treated with 30 000 to 40 000 I U per day and in two subjects with vitamin D-resistant rickets treated with 150 000 to 500 000 I U per day. Thus it appears that under the influence of vitamin D calcium and phosphate are mobilized from one part of bone to be deposited in another.

As Nicolay en and Leg Larsen have pointed out [111] it is both important and difficult in attempting to establish the actions of vitamin D to distinguish between effects of vitamin D on calcium absorption and direct effects on bone. The same problem exists in the study of the pathologic physiology of vitamin D-resistant rickets. One cannot say from available evidence whether the response of the bone to normal levels of vitamin D would be normal if the serum concentrations of calcium and phosphate were maintained at normal levels for prolonged periods by means other than very large doses of vitamin D.

The similarity of the changes in metaphysis and diaphysis to those seen in osteochondrodystrophy raises the possibility that this bony abnormality is another congenital presumably inherited defect in these patients and not directly related to hypophosphatemia or to vitamin D. The lesion could also be a direct effect of parathyroid overactivity although the changes described have not been entirely typical.

### POSSIBLE MECHANISMS OF PATHOGENESIS AND QUESTIONS STILL UNANSWERED

This discussion of pathogenesis of familial hypophosphatemia and vitamin D-resistant rickets will be focused on the two abnormalities which the authors consider to be the most characteristic features of the disorder: hypophosphatemia and decreased renal tubular reabsorption of phosphate. The other abnormalities thus far identified—low gastrointestinal absorption of calcium and the bony abnormalities—will be discussed in so far as they bear upon the pathogenetic possibilities to be presented. A final decision about mechanism cannot now be made.

#### *A Genetically Determined Intrinsic Renal Tubular Defect in Reabsorption of Phosphate*

This possibility offers the simplest explanation for the occurrence of hypophosphatemia and decreased renal tubular reabsorption of phosphate. Consistent with this hypothesis are the facts that hypophos-

phosphatemia has a simple genetic pattern, and that there is decreased tubular reabsorption of phosphate in all instances in which it has been looked for. One may propose therefore an isolated defect in the tubular mechanism responsible for the transport of phosphate. This explanation originally suggested by Robertson, Harris and McCune [2] has also been offered by Dent, Fanconi and others [17, 27, 91, 134-136].

Certain qualifications must be made for such a proposed intrinsic tubular defect. First, reabsorption of phosphate is only *decreased*, not absent. As the data of Tables 36-2 and 36-3 show, these patients do have a significant rate of reabsorption of phosphate which is about half of normal. It is possible that there may be two "sites" or mechanisms by which phosphate is reabsorbed, only one of which is absent or blocked in familial hypophosphatemia.

A second qualification is that the tubular phosphate-reabsorbing mechanism which is still intact in these patients is probably responsive to parathyroid hormone. This is discussed below in the next section.

The response of the phosphate reabsorptive mechanism in subjects with vitamin D-resistant rickets to other agents which diminish tubular reabsorption of phosphate has not been studied. It is of interest that all agents whose effect on  $Tm_p$  has been quantitatively determined (parathyroid hormone, cortisone, glucose, acetoacetate, bicarbonate) lower the  $Tm_p$  to approximately the range seen in familial hypophosphatemia. Table 36-5 summarizes the available data. It is possible that all these agents affect (perhaps indirectly) one pathway for phosphate transport, while a second mechanism is independent of their effects. This possibility might be studied by determining the effects of combinations of the agents on  $Tm_p$  in normal and in affected persons.

Some features of this disease are not readily explicable by a simple renal tubular defect, viz. the decreased gastrointestinal absorption of calcium (and phosphate) and the metaphyseal and diaphyseal abnormalities in the bone. It is possible that a (partial) defect in intestinal absorption of phosphate, analogous to the renal tubular defect, could be present. However, the patient of Albright et al. [1] absorbed over 80 per cent of ingested phosphate when on a low calcium intake. Gunther et al. [110] in one patient with vitamin D-resistant rickets found all but 5 per cent of ingested radiophosphorus absorbed when it was not given with food. Similar measurements have not been reported in familial cases.

Fanconi [135] has proposed a sequence of events as follows: the decreased tubular reabsorption of phosphate is the primary determinant of the low serum phosphorus concentration. Accordingly, deposition of bone salts in osteoid tissue is subnormal. Calcium absorption is in some way related to the rate of deposition of calcium in bone and is thus also low. Consistent with this proposal is the fact that healing of the bone disease and increased calcium absorption are initiated by large in-

travenous or oral loads of phosphate [119]. The last link in the above sequence, the dependence of calcium absorption upon rate of bone deposition has not been established.

TABLE 36-5 EFFECTS OF VARIOUS SUBSTANCES ON MAXIMAL TUBULAR REABSORPTION OF PHOSPHATE

| Ref<br>e ce | S t e c c  | S u b j e c t | T <sub>mp</sub> before |                          | T <sub>mp</sub> after |            |
|-------------|--|---------------|------------------------|--------------------------|-----------------------|------------|
|             |  |               | Mean                   | (I a n g )               | Mean                  | (I a n g ) |
| [ 4 ]       | Parathyroid extract intramuscularly for 3-5 days | Man           | 114                    | (110-118)                | 68                    | (31-101)   |
| [ 1 ]       | Cortisone intravenously                          | Dog           | 140                    |                          | 66                    |            |
| [ 10 ]      | Cortisone orally or hydrocortisone intravenously | Man           | 170                    | (100-175)                | 96                    | (61-120)   |
| [ 10 ]      | Diethylstilbestrol for 14-21 days                | Man           | 134                    | (91-104)                 | 100                   | (67-120)   |
| [74]        | Acetate loading                                  | Dog           | 116                    | (110-124)                | 77                    | (64-81)    |
| [ 3 ]       | Glucose loading                                  | Dog           | 118                    | (103-134)                | 73                    | (61-85)    |
| [ 1 ]       | Glucose loading                                  | Dog           | 115                    | (101-129)                | 65                    | (50-74)    |
| [ 8 ]       | NaHCO <sub>3</sub>                               | Acetate dog   | 9                      | (8-17)                   | 4*                    | (4-50)     |
| [76]        | Respiratory alkalosis                            | Dog           | 8                      | (8-13)                   | 64                    | (64-71)    |
| [ 5 ]       | Alanine or glycine                               | Dog           | 193<br>157             | (184-197)†<br>(144-151)‡ | 89‡<br>53‡            |            |

Expressed in micromoles per 100 ml glomerular filtrate

† Fasting following carbohydrate diet

‡ Fasting following meat diet

\* Based on textual statement that the T<sub>mp</sub> fell to about 75 per cent of control value

### *Hyperparathyroidism Secondary to a Defect in Gastrointestinal Absorption of Calcium*

On the basis of their studies on the first patient in whom the diagnosis of vitamin D-resistant rickets was made Albright et al [184] proposed that the primary event is decreased absorption of calcium because of resistance to vitamin D. This leads to a low serum calcium concentration which in turn stimulates parathyroid secretion. Increased parathyroid hormone activity decreases tubular reabsorption of phosphate and hypophosphatemia occurs. Secondary to the hypophosphatemia (as Albright proposed) or due to another direct action of parathyroid hormone on bone (as subsequent work has shown [137-139]) resorption of calcium from bone occurs and raises the serum calcium concentration. If the compensatory overactivity of the parathyroid glands should be just sufficient the final results of this sequence could be normal serum

phthemia has a simple genetic pattern and that there is decreased tubular reabsorption of phosphate in all instances in which it has been looked for. One may propose therefore an isolated defect in the tubular mechanism responsible for the transport of phosphate. This explanation originally suggested by Robertson, Harris and McCune [2] has also been offered by Dent, Lanconi and others [17, 27, 91, 134-138].

Certain qualifications must be made for such a proposed intrinsic tubular defect. First, reabsorption of phosphate is only *decreased* not absent. As the data of Tables 36-2 and 36-3 show, these patients do have a significant rate of reabsorption of phosphate which is about half of normal. It is possible that there may be two "sites" or mechanisms by which phosphate is reabsorbed, only one of which is absent or blocked in *familial hypophosphatemia*.

A second qualification is that the tubular phosphate reabsorbing mechanism which is still intact in these patients is probably responsive to parathyroid hormone. This is discussed below, in the next section.

The response of the phosphate reabsorptive mechanism in subjects with vitamin D-resistant rickets to other agents which diminish tubular reabsorption of phosphate has not been studied. It is of interest that all agents whose effect on  $Tm_p$  has been quantitatively determined (parathyroid hormone, cortisone, glucose, acetoacetate, bicarbonate) lower the  $Tm_p$  to approximately the range seen in *familial hypophosphatemia*. Table 36-5 summarizes the available data. It is possible that all these agents affect (perhaps indirectly) one pathway for phosphate transport while a second mechanism is independent of their effects. This possibility might be studied by determining the effects of combinations of these agents on  $Tm_p$  in normal and in affected persons.

Some features of this disease are not readily explicable by a simple renal tubular defect, viz., the decreased gastrointestinal absorption of calcium (and phosphate) and the metaphyseal and diaphyseal abnormalities in the bone. It is possible that a (partial) defect in intestinal absorption of phosphate analogous to the renal tubular defect could be present. However, the patient of Albright et al. [1] absorbed over 80 per cent of ingested phosphate when on a low calcium intake. Gunther et al. [115] in one patient with vitamin D-resistant rickets found all but 5 per cent of ingested radiophosphorus absorbed when it was not given with food. Similar measurements have not been reported in familial cases.

Lanconi [180] has proposed a sequence of events as follows: the decreased tubular reabsorption of phosphate is the primary determinant of the low serum phosphorus concentration. Accordingly, deposition of bone salts in osteoid tissue is subnormal. Calcium absorption is in some way related to the rate of deposition of calcium in bone and is thus also low. Consistent with this proposal is the fact that healing of the bone disease and increased calcium absorption are initiated by large in-

Lamy et al [21] in one child with nonfamilial vitamin D-resistant rickets or a variant of the Lianconi syndrome found that near the end of a 3 hr calcium infusion the urine became phosphate free (and aminoaciduria previously present also disappeared). Fraser et al have made similar observations on phosphate excretion with more prolonged calcium infusions in two children with vitamin D-resistant rickets: subcutaneous administration of parathyroid extract then restored the phosphaturia [144, 145]. Field and Reiss [146] found that the %TRP rose from low to normal levels in 12 affected members of three families with this disorder. However, since serum phosphorus concentration also remained below normal in these studies, the amount of phosphorus actually being reabsorbed by the tubules remained low. Only by raising serum phosphorus to normal or preferably to a level which clearly saturates tubular reabsorptive capacity, at the time of a calcium infusion, could one hope to differentiate unequivocally between the role of the parathyroid glands and that of an underlying tubular defect. In one such study in an affected adult member (IV-5) of the C kindred of Winters et al [8]  $Tm_p$  in the sixth hour of calcium infusion (13 mg per kg over 6 hr) was low and not significantly different from two previous measurements of  $Tm_p$  in the same subject (Table 36-3). Further studies of this type are needed in familial and nonfamilial instances of the disorder; it is entirely possible that more than one mode of pathogenesis exists.

Parathyroid extract given *intravenously* to several patients with this disorder has produced variable increases in phosphate excretion [17, 28, 29]. It is now known, as best demonstrated by Hiatt and Thompson [34], that intravenously administered parathyroid extract increases glomerular filtration rate to such a degree that no interpretation can be made about a change in tubular reabsorption.

The evidence that hyperparathyroidism is *not* a prominent feature of familial hypophosphatemia is inferential. In patients with hypophosphatemia only, it seems improbable that a defect in calcium absorption could be so delicately balanced by parathyroid overactivity that serum calcium concentration would be maintained at a normal level throughout life without any signs of hyperparathyroidism other than increased phosphate excretion. The absence of clinical or radiologic evidence of bone resorption in the asymptomatic patients is additional evidence against the presence of chronic hyperparathyroidism. Histologic examination of specimens of bone would be most helpful.

It is impossible to decide at present whether the hypophosphatemia and decreased renal tubular reabsorption of phosphate are due to an intrinsic renal tubular defect or to hyperparathyroidism secondary to an intestinal defect in absorption of calcium. Several lines of investigation which might help to clarify this question have already been indicated. It is conceivable that parathyroid hormone depresses the reabsorption

calcium (and normal ionized calcium [62]) low serum phosphorus concentration and diminished renal tubular reabsorption of phosphate which are the findings of patients with vitamin D-resistant rickets

If Albright's scheme is correct, one would expect histologic evidence of parathyroid hyperplasia and evidence of bony changes characteristic of hyperparathyroidism. Albright et al [1] actually found hyperplastic changes in one parathyroid gland removed from their patient. Frame and Smith [136] made a similar observation. No parathyroid glands have been examined in patients with familial histories of this disease. As already noted in the section on the bony abnormality, the changes that have occasionally been found in diaphyseal and metaphyseal regions have been interpreted as similar to those of experimental hyperparathyroidism. Furthermore in a number of patients particularly those reported by Holt [19] the lamina dura has been absent. This finding has been observed more commonly in hyperparathyroidism than in any other type of bone disease but it has not been looked for extensively in other types of rickets and osteomalacia. It has been noted in certain cases of osteomalacia due to steatorrhea and to renal tubular acidosis [90].

In the single report of assay of the serum for parathyroid hormone activity in a patient who probably had resistant rickets Highman and Hamilton [140] found a higher than normal value by their calcium mobilization test. Such a test is only a crude measure of a single parathyroid function and one which may not be correlated with the effects on phosphate excretion [141-143].

Renal tubular reabsorption of phosphate in vitamin D-resistant rickets is probably responsive to changes in parathyroid activity. Albright et al [1] found in their patient that administration of parathyroid hormone (500 units a day intramuscularly for 3 days) caused an increase in phosphate excretion. This dose and route in normal man increase phosphate excretion by decreasing tubular reabsorption without any change in glomerular filtration rate [54].

Suppression of parathyroid function has been the interpretation given to the results of intravenous calcium infusions in normal subjects. Phosphate excretion usually falls following the infusion presumably because of suppression of parathyroid function by the elevated serum calcium concentration. In two affected adult members of the "E" kindred of Winters et al [8] intravenous calcium loading caused a 30 to 60 per cent decrease in phosphate excretion which is similar to what occurs in normal persons [63-65-66]. These results suggested that the kidneys of the patients do respond to changes in parathyroid activity however in the presence of their low levels of serum phosphorus it might have been expected that the urine would have become phosphate free if increased parathyroid activity were the only factor affecting phosphate excretion.

Others have observed more striking responses to calcium infusions

to normal when vitamin D is given. Serum citrate concentration rises to supernormal levels when vitamin D intoxication is produced in patients with vitamin D-deficiency rickets and vitamin D-resistant rickets [39 89 90]. The effects of vitamin D on citrate metabolism are apparently not necessarily related to its effects on promoting calcification of osteoid. Cortisol when given to vitamin D-deficient rats prevents the rise in citrate concentration of serum and bone which vitamin D usually produces but does not block the action of vitamin D in raising the serum calcium and phosphate concentrations and healing of the rickets [100]. Conversely when vitamin D-deficiency rickets in rats is healed by giving calcium and phosphate but no vitamin D the citrate content of bone and serum remains low [133]. It would be interesting to know whether the healing of vitamin D-resistant rickets initiated by phosphate administration or immobilization is accompanied by changes in citrate content of bone or serum.

#### *Other Possible Mechanisms of Pathogenesis*

Kodicek [151] in 1956 gave a preliminary report of abnormal  $\Delta^4$  3 ketosteroids in the urines of seven patients with vitamin D-refractory osteomalacia unrelated to vitamin D administration. Cortisone depresses tubular reabsorption of phosphate in the dog and in man under some circumstances and may decrease intestinal absorption of calcium. A related steroid might well have a more marked effect on these functions and be casually related to this disease. The production of such an abnormal steroid could be genetically determined.

It has also been proposed that there is a generalized abnormality in the intermediary metabolism of phosphate perhaps affecting all body cells [8 29 29]. Studies related to this possibility have thus far been applied to erythrocytes only [102-154] where decreased concentrations of organic phosphate compounds have been found in experimental and naturally occurring rickets. It appears likely that this finding is a reflection of a generally low body content of labile phosphorus rather than the result of an abnormality in intermediary metabolism due to lack of vitamin D [155]. Studies of this type have not been made in patients with familial hypophosphatemia and vitamin D-resistant rickets.

An observation which may indicate a more specific abnormality in vitamin D-resistant rickets is that of Rupp [156] that erythrocytes of such a patient incorporate more radioactive phosphorus into creatine phosphate and less into adenosine triphosphate than erythrocytes of normal subjects.

#### *Summary of Possible Mechanisms of Pathogenesis*

The development of the abnormalities in familial hypophosphatemia and vitamin D-resistant rickets is not yet understood. The presence of a simple genetically determined renal tubular defect in reabsorption of



of phosphate of all tubules more or less equally, whereas in familial hypophosphatemia an intrinsic tubular defect might involve some tubules to a greater extent than others. In the latter circumstance an increased "splay" area in the titration curve might be detectable.

#### *An Abnormality in the Absorption of Vitamin D*

There is little direct evidence for an abnormality in the absorption of vitamin D in vitamin D-resistant rickets. The blood level of the vitamin as measured by the bioassay technique of Warkany is normal prior to treatment with massive doses, and markedly elevated afterwards [20, 116, 120]. In addition the bone disease is not prevented or cured by parenterally administered conventional doses of vitamin D [1, 12, 20, 24, 28, 29] nor is ultraviolet light effective in curing rickets of the resistant type [1, 10, 21, 115, 116, 147].

#### *An Abnormality in the Action of Vitamin D*

The fact that large doses of vitamin D will cure the rickets cannot be considered *prima facie* evidence that the cause of the disorder is a resistance to normal amounts of the vitamin. It has already been pointed out that healing of the rachitic bone lesions is initiated by supplying adequate phosphate without additional vitamin D. The action of massive doses of vitamin D (or comparable doses of dihydrotachysterol) on intestinal absorption of calcium is similar to that seen in persons who do not have rickets of either type.

Nevertheless the possibility must be considered that these patients do have a resistance to vitamin D at the level at which it exerts its action in normal individuals. This "resistance" would presumably be inborn in the familial cases and acquired in the sporadic instances. Support for this view comes from the descriptions of patients who have been only partially resistant and are cured by doses of intermediate size [5, 148]. Other patients have developed increasing resistance to vitamin D and have required larger and larger doses [149]. Certain patients have seemed to respond to dihydrotachysterol when they did not respond to vitamin D<sub>2</sub> or D<sub>3</sub> in large doses [147]. These last observations suggest a resistance to specific sterols as has been described for some patients with hypoparathyroidism [149]. No familial case of sterol specific resistance has been described.

Tobler et al. [29] refer to preliminary studies by Flückiger of a serologic reaction to vitamin D, found in the serum of patients with vitamin D-resistant rickets but not in the serum of normal persons. Further evidence on this point is needed.

Clues to the role of vitamin D in familial hypophosphatemia and vitamin D-resistant rickets might come from studies of citrate metabolism. In vitamin D-deficiency states a low content of citrate is found in bone, intestines, kidney, serum and urine. These levels promptly return

hypophosphatemia. A clear generation to generation transmission of the trait emerged and there was no evidence of *skipping*.

The same result was found not only in other portions of this same kindred but also in three additional unrelated kindreds studied subsequently [9]. On the basis of these studies which include a total of 78 hypophosphatemic persons, the following conclusions were drawn:

1. All of the 40 persons who had skeletal disease or deformities were hypophosphatemic.

2. An additional 38 individuals were hypophosphatemic but had no definite evidence past or present of skeletal disease.

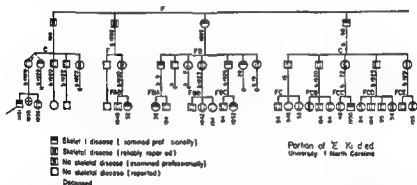


Fig 36-6 A portion of the pedigree of the F kindred of Winters et al [9] scored according to the presence of skeletal disease. No clear-cut pattern of inheritance can be identified.

3. In these 38 were included all 11 instances of proved or probable skipping which were scored when skeletal disease was used as the criterion for scoring.

4. Hypophosphatemia was inherited in a clear and simple fashion: each hypophosphatemic person had one hypophosphatemic parent in all of the 50 instances where the requisite data were available.

5. No hypophosphatemic person had more than one hypophosphatemic parent in a number of matings where both parents were examined.

Brothers and sisters of hypophosphatemic persons included both normal and affected persons in approximately equal numbers.

On the basis of these results it is apparent that hypophosphatemia is inherited as a dominant trait with a high degree of penetrance (perhaps not complete penetrance in young girls as indicated below) but the transmission of hypophosphatemia even if fully penetrant is insufficient to produce overt bone disease. There can be little doubt that hypophosphatemia is an important but not the sole determinant of bone disease.

These studies also indicate that the mode of transmission is not autosomal but rather is sex-linked. This conclusion is based upon the authors

phosphate would account very well for the findings in those patients with asymptomatic hypophosphatemia but any attempt to explain the frequent additional findings—the bony abnormalities and defective absorption of calcium—on the basis of a renal tubular defect immediately becomes speculative. The possibility that the primary defect is in absorption of calcium, with an associated secondary hyperparathyroidism has been neither proved nor disproved but seems an unlikely explanation for the existence of asymptomatic hypophosphatemic individuals in the involved families. There is no direct supporting evidence for the proposal that there is an abnormality in the metabolism of vitamin D or a resistance to its action at normal levels. The action of large doses in curing the rachitic lesions can be explained in terms of the action of such doses in normal persons. The evidence for a disturbance in steroid metabolism may be a promising lead.

### GENETIC ASPECTS

There is a definite tendency for vitamin D-resistant rickets to occur in families. In 65 adequately documented cases in the literature prior to 1958 over half had a positive family history [8]. From studies of small pedigrees in which affected individuals were scored on the basis of overt bone disease several workers [9-11] advanced the hypothesis that the disease was transmitted as an autosomal dominant trait. However the overt form of the disease had been reported more frequently in females than in males. Analysis of the distribution of normal and affected females and males among the progeny of affected persons in the earlier studies revealed a deviation from the 1:1:1:1 ratio predicted by the autosomal dominant hypothesis. The primary cause of this deviation was an excess of affected females [8]. These considerations suggested either that there is a strong influence of sex on the expression of an autosomal trait or that some other mode of transmission is operative.

Winters et al. [8] reinvestigated the genetic aspects of the disease in a large North Carolina kindred (E kindred). Individuals were scored not only on the basis of skeletal disease but also on the basis of the level of serum inorganic phosphorus (see Hypophosphatemia and the Abnormality in Renal Excretion of Phosphate). Comparison of these two methods of scoring revealed that the latter is a superior discriminant for the trait. This point is illustrated in Figs. 36-6 and 36-7 where a portion of the E kindred is reproduced. In Fig. 36-6 individuals were scored as affected on the basis of clinical, radiographic or historical evidence of active rickets (children) or postrachitic deformities (adults). Using this method there was no clear cut pattern of inheritance and several instances of skipping of a generation were noted. This may be contrasted with the findings in Fig. 36-7 where scoring was based upon

own studies of four kindreds. In the *classified* progeny of affected fathers all 16 males were normal while all 20 of the females were affected (Table 36-6). The chance of this distribution occurring on the basis of an autosomal dominant mechanism is virtually nil.

A sex-linked dominant mechanism implies that the abnormal gene resides on the X-chromosome. An affected female is therefore heterozygous ( $X^*X$ ) whereas an affected male lacking the normal allele is hemizygous ( $X^*Y$ ). Since the male progeny of an affected male receive their Y-chromosome from their fathers they would all be normal. On the other hand female progeny of an affected male receive the single  $X^*$  chromosome of the father and are therefore all affected. The sex-linked dominant hypothesis also predicts that half the progeny of an affected

TABLE 36-6 DATA ON FOUR KINDREDS OF HYPOPHOSPHATEMIC PATIENTS

| Sex of progeny                                 | Affected | Normal | Total |
|--|----------|--------|-------|
| Classified progeny of hypophosphatemic fathers |          |        |       |
| Male   | 0        | 16     | 16    |
| Female   | 20       | 0      | 20    |
| Total  | 0        | 16     | 36    |
| Classified progeny of hypophosphatemic mothers |          |        |       |
| Male   | 20       | 13     | 33    |
| Female   | 10       | 24     | 34    |
| Total  | 30       | 37     | 67    |

SOURCE: R. W. Winters et al. [8] and J. M. Graham et al. [9]

female should be normal and half affected without regard for sex. Data on this point are also shown in Table 36-6 and demonstrate a significant deviation from the expected distribution due primarily to an excess of normal daughter. Although the complete explanation for this unexpected distribution is not clear it is noteworthy that many of the daughters scored as normal were in the first decade where the discriminant for the diagnosis of hypophosphatemia gives a larger rate of false negative diagnoses than at any other age group in either sex [9-30].

The sex-linked dominant hypothesis not only explains more adequately than the autosomal dominant mechanism the results of the authors' own studies but also offers a better explanation for the previously reported genetic data. No pedigree published by other workers is sufficiently large to reject the autosomal hypothesis with confidence but all are equally well explained by the sex-linked dominant mechanism. The latter hypothesis also accounts for the excess of affected females in families previously described. The clear rejection of the sex-linkage would be a documented instance of male to male transmission. Although there is a

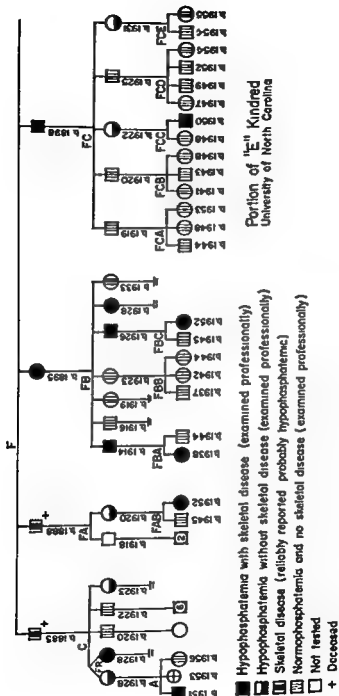


Fig 36-7 The same pedigree as that shown in Fig 36-6 scored according to the presence of hypophosphatemia (generation to generation transmission with no evidence of skipping can now be seen)

variety. A recently studied sporadic case [107] was seen in a severely affected female who showed no clinical or chemical features distinguishing her from patients with the sex-linked variety of the disease. Both parents and many relatives of this patient had no clinically or chemically identifiable related disease. The disease in this and similar patients could be the result of a new mutation or of a recessive trait or could represent a phenocopy.

## TREATMENT

The treatment of vitamin D-resistant rickets is highly individualized; it must be based on recognition not only of the age and growth potential of the patient and the severity of his skeletal disease but also of the very real risks of hypervitaminosis D.

### Vitamin D Therapy

It is in children with overt bone disease that the need for therapy is most pressing. The general plan of therapy is to start with a dose of vitamin D<sup>1</sup> of 25,000 to 50,000 I.U. daily. This is then increased by increments of 25,000 I.U. per day at intervals of 1 month to 6 weeks. Increases are determined by chemical evidence (principally a falling level of alkaline phosphatase in the serum) or radiographic evidence of healing. The dosage necessary to effect clear-cut healing is variable but is generally of the order of 150,000 to 250,000 I.U. per day. Once healing has been initiated, there is no assurance that the dose may remain constant; subsequent alterations are often necessary in order to avoid reactivation on the one hand or cumulative vitamin D poisoning on the other.

The risks of hypervitaminosis D are considerable. In some patients the effective therapeutic dose lies in the frankly toxic range. In order to avoid serious degrees of hypercalcemia, continuous medical supervision is mandatory and should include a careful search for signs and symptoms of hypercalcemia, serial measurements of serum calcium, and frequent urinalyses and estimations of blood urea nitrogen. Mild but significant hypercalcemia is often unaccompanied by objective signs and clear-cut symptoms. The Sulkowitch test for calcium in the urine is not of great value in the early recognition of vitamin D poisoning, but it may be

<sup>1</sup> Vitamin D (calciferol) is most widely used. Vitamin D is also effective as is dihydrotachysterol (A.T.10) but the latter two agents are not preferred because of expense and relative unavailability. The fact that dihydrotachysterol in doses which increase the intestinal absorption of calcium [8-11] will heal vitamin D-resistant rickets whereas its effectiveness in healing ordinary rickets is poor compared with vitamin D is further evidence for the view that the corrective action of these sterols in vitamin D-resistant rickets is a non-specific effect on calcium absorption. Consistent with this view is the healing action of dihydrotachysterol in vitamin D-deficiency rickets due to a low-calcium diet in rats [153].

single report suggesting this occurrence [10] the mother of the affected boy was not examined for hypophosphatemia.

In the first kindred studied by Winters et al [7, 8] a marked difference was observed between heterozygotes and hemizygotes with respect to the severity of bone disease. Hypophosphatemic males nearly always had obvious rickets or postrachitic deformities while hypophosphatemic females tended to have minimal skeletal involvement. In general this difference has been confirmed in the three pedigrees subsequently studied [9]. A summary of these findings appears in Table 36-7. Although exact rating of severity of bone disease is admittedly difficult, there can be little doubt about the tendency of males to be more severely affected than females.

TABLE 36-7 CLASSIFICATION OF HYPOPHOSPHATEMIC PERSONS BY SEXES ACCORDING TO SEVERITY OF BONE DISEASE

|         | Int | Mild | Moderate or severe | Total |
|---------|-----|------|--------------------|-------|
| Males   | 2   | 1    | 27                 | 30    |
| Females | 36  | 2    | 10                 | 48    |
| Total   | 38  | 3    | 37                 | 78    |

SOURCE: From studies of four kindreds by R. W. Winters et al [8] and J. B. Graham et al [9]. Classification made according to the general criteria used in [8].

In searching for an explanation for these differences statistical comparison of the levels of serum phosphorus was made between the various subgroups of heterozygotes and hemizygotes in the E kindred. In order to make any valid comparison of this type the data were age adjusted. Males in various age groups had lower levels of serum phosphorus than females in the corresponding groups. These differences although statistically significant were small. The largest was 0.47 mg per 100 ml between affected male and female children. In view of the uncertainties about the method of age adjustment the significance of these differences must remain uncertain.

The relative immunity of the female from severe skeletal disease has at least two possible explanations: (1) the normal allele may modify the action of the abnormal gene so that the female escapes severe bone disease because of heterozygosity; (2) the relative immunity of the female is a factor operative in that sex independent of gene dosage. Since there is no proved instance of a homozygous female this problem remains unresolved.

Some previous authors have reported cases of resistant rickets in which both parents were clinically normal (cf Winters et al [8] for review). In no instance have the parents been examined chemically.

Not all cases of vitamin D-resistant rickets are of the sex-linked

The treatment of osteomalacia should proceed along the lines outlined above for the treatment of affected children

In affected adults without osteomalacia the problems are those of residual deformities and the secondary arthritic complications Treatment with vitamin D is not indicated for these problems

#### *Treatment of Asymptomatic Hypophosphatemia*

There would seem to be little reason to treat patients with asymptomatic hypophosphatemia If it could be shown that treatment with vitamin D improved the growth of such children then therapy might be considered

### PREVENTIVE MEASURES

Once the mode of inheritance of the trait has been determined in a particular family the affected individuals and their spouses should be fully informed of the nature of the transmission of the gene as well as the chances and probable consequences of having a child with the disease Potentially affected progeny of such a mating should then be studied as early as practicable in order to establish the genotype on the reasonable assumption that institution of therapy may prevent serious degrees of deformity As noted earlier (see Hypophosphatemia above) the detection of hypophosphatemia is difficult in a young infant The diagnosis can be made or excluded with confidence only by obtaining multiple determinations

### SUMMARY

1 Familial hypophosphatemia and vitamin D-resistant rickets is a disorder in which the most distinctive features are hypophosphatemia and diminished renal tubular reabsorption of inorganic phosphate

2 The inheritance pattern is that of sex linked dominance with complete penetrance

3 The rickets or osteomalacia which may develop is not responsive to usual therapeutic doses of vitamin D but can be healed with massive doses of the vitamin

4 Children with bony involvement probably have abnormally low absorption of calcium from the gastrointestinal tract This responds only to large amounts of vitamin D a response similar to the effects of large doses of vitamin D on intestinal absorption of calcium in normal individuals

5 Healing of the rickets can be initiated by measures which do not involve vitamin D such as immobilization or administration of large amounts of phosphate It thus appears that the bony involvement is secondary to the disorder in phosphate metabolism rather than to a lack of a direct action of vitamin D on bone



helpful to have the parents perform the test regularly to focus their attention upon this ever present problem

In most instances careful adjustment of the dosage of vitamin D is successful in controlling the active rachitic process. As healing occurs the level of alkaline phosphatase in the serum falls to normal. Phosphorus balance becomes positive but serum phosphorus does not often rise to clearly normal values. At first glance this may seem to contradict the hypothesis that hypophosphatemia is a dominant determinant of bone disease. However the experiments of Yendt and Howard [159] provide an explanation. They found that transient increases in the level of phosphate in rachitic rats may be sufficient to initiate calcification, which continues even though the ambient concentration of phosphate is again reduced to levels associated with rickets.

Healing of the rickets is often associated with some improvement in the severity of the deformities although in cases with serious degrees of deformity subsequent osteotomy may be indicated. Surgical correction should be undertaken only after the active process is fully controlled. *Before and during the period of immobilization the dosage of vitamin D must be greatly reduced or eliminated and then reinstituted with mobilization.*

Another major unsolved problem in children is that although the rachitic process may be optimally controlled by vitamin D, the expected increase in the rate of linear growth does not often occur and the patients remain dwarfed. The possibility that continuing hypophosphatemia limits linear growth independently of its effect upon the rachitic lesion deserves further study.

### *Other Therapeutic Measures*

It has been shown [114] that adding inorganic phosphate to vitamin D therapy may increase phosphate and calcium retention restoring the serum phosphorus concentration to normal. The administration of phosphate will initiate healing even in the absence of vitamin D [119]. However after more extended observations Fraser has concluded that long term phosphate therapy has no proved place in the management of vitamin D-resistant rickets and may even be harmful [95].

### *Treatment of Affected Adults*

The rarity of overt osteomalacia in adults with hypophosphatemia and a history of vitamin D-resistant rickets is remarkable. In the large kindred studied by Winters et al [8] there were 23 adequately studied hypophosphatemic adults. Only 2 of these had evidence of osteomalacia in the form of single pseudofractures. A few affected adults with serious degrees of osteomalacia have been reported by others [5, 28, 91, 92, 101].

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6 One of the leading explanations proposed for the disease is that there is an intrinsic defect in the function of the renal tubular cells. If so the defect is highly specific for it is not associated with any other known tubular defect. Furthermore the aminoaciduria which accompanies vitamin D-deficiency rickets is not found in individuals with familial hypophosphatemia.

7 The second leading explanation is that there is overactivity of the parathyroids, secondary to diminished intestinal absorption of calcium which depresses renal tubular reabsorption of phosphate. If so, the compensatory adjustment is remarkably exact for serum calcium concentration is almost always normal and hyperparathyroidism is not detectable clinically.

8 Studies of the quantitative aspects of tubular reabsorption of phosphate in relation to parathyroid function to the action of vitamin D and to the reabsorption of other substances which may affect phosphate reabsorption are needed to clarify the fundamental abnormality in familial hypophosphatemia.

9 From genetic considerations it is possible to predict individuals likely to develop the disorder, and to take the necessary steps toward early detection and prevention of the deforming features of this disease.

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## Chapter 37

### The Syndrome of Osteomalacia, Renal Glycosuria, Aminoaciduria, and Hyperphosphaturia (The Fanconi Syndrome)

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Alexander Leaf

The main features of this syndrome upon which there is general agreement are rickets or osteomalacia resistant to vitamin D in usual doses, glucosuria, generalized aminoaciduria, and hyperphosphaturia in spite of normal or reduced plasma concentrations of the  $\alpha$  constituents and usually chronic acidosis and hypopotassemia. Beyond these cardinal features one finds much disagreement and confusion in the literature pertaining to this rare disturbance. Recent awareness that multiple etiologies may reproduce these cardinal features and that each feature may occur separately or in various incomplete combinations indicates that the appellation *syndrome* rather than *disease* is appropriate. To avoid ambiguity in the present writing eponyms will be avoided and the italicized term *syndrome* will be used to apply to the complete constellation of features as defined above.

#### HISTORICAL RESUME

The history of the recognition of this *syndrome* has been confused by uncertainty as to just which features it encompasses and which eponyms should be applied to it. Not all the features recognized today as part of the *syndrome* were appreciated by earlier workers. Their case reports therefore seem fragmentary and incomplete making it difficult to trace the historic lineage accurately.

In 1903 Abderhalden [1] found by chemical assay excessive amounts of cystine in various tissues at post mortem examination of a 21 month old Swiss infant who died of inanition. Crystals identified as cystine

were visible macroscopically in the liver and spleen. Two siblings had died previously of a similar ailment, and the urine of two living siblings contained excessive cystine, as did to a lesser extent the urines of the father and paternal grandfather. He thus labeled the disorder a familial cystine diathesis.

In 1924 Lignac [2-3] a Dutch pediatrician described three similar cases in children 14 months old and 2 and 3 years of age. Clinically all three had severe rickets, renal disease, dwarfism, and progressive wasting. Pathologically all showed widespread deposits of cystine in the tissues and nephritis, and one child reportedly had cystine stones in the renal pelvis and ureter. Lignac also noted the familial incidence of the disease, as a sister of one of his patients was afflicted. Two further cases were reported from England by Russel and Barrie [4] in 1936. One child had rickets with a low normal serum phosphate concentration but no cystine crystalluria. Both he and the second child probably died in uremia, since post mortem examination revealed extensive glomerular and tubular damage as well as cystinosis. The following year Beumer [5] added albuminuria and glycosuria to the clinical picture and demonstrated in his case that the nonprotein nitrogen level became elevated only terminally. Thus, cystine storage disease came to be recognized.

Meanwhile in 1931 Fanconi [6] briefly described rickets and stunted growth in a child with albuminuria and glycosuria. In 1933 deToni [7] reported a dwarfed child with vitamin D-resistant rickets and spontaneous fractures who showed a low serum phosphorus concentration, acidosis, glycosuria, and albuminuria. With a similar case reported by Debré [8] in 1934, the one of deToni and two more of his own, Fanconi [9-10] in 1936 established a syndrome of nephrotic glycosuric dwarfism with hypophosphatemic rickets. Beumer and Wepler [11] in 1937 surmised that cystinosis and the Fanconi syndrome were identical, and this seemed proved when at necropsy one of Fanconi's original cases showed cystine deposits in his tissues [12]. The extreme form of this view was supported by Bickel and associates [14] who concluded that Lignac's disease (cystine storage disease) and Fanconi's syndrome of childhood (nephrotic glycosuric dwarfism) are one and the same disease, and at the present time this disease cannot be identified with similar syndromes in which cystine storage has been excluded. Though it is generally accepted today that the two conditions are similar, this point of view is opposed by deToni [13] who believes that when cystinosis and the syndrome of renal glyco-amino-phosphaturia coexist, the latter probably represents a complication of the former. DeToni would therefore create two separate syndromes: the Alderhalden-Kaufmann-Lignac syndrome to designate cystine storage disease, and the deToni-Debré-Fanconi syndrome to designate rickets or osteomalacia with renal glyco-amino-phosphaturic diabetes. The nomenclature is further confused by the use of various

combinations of eponyms for various features of the syndrome. Many case reports of this *syndrome* are summarized in the monographs of McCune, Mason, and Clarke [15], Bickel, Smallwood, Smellie, and Hickmans [14], and Eberlein [16].

The aminoaciduria in the *syndrome* was first suspected by Fanconi [10] when he found an increased organic aciduria. McCune, Mason and Clarke [15] were the first to demonstrate that the organic aciduria was in fact largely attributable to amino acids.

It has become evident that the *syndrome* is not restricted to infancy or childhood but may present initially in adult life with the same cardinal features. Cystine deposits in the tissues have not as yet been described in the adult cases although they are commonly an associated finding in the childhood form. The adult form of the *syndrome* has recently been reviewed by Walsh and Lingle [17], who in 1957, collected 18 cases from the literature.

## CLINICAL FEATURES

In the infant the disease characteristically begins after a normal postnatal period of 4 to 6 months. Failure to grow, often bouts of vomiting and unexplained fever, a severe form of rickets resistant to the usual doses of vitamin D, chronic acidosis, polyuria and dehydration dominate the clinical picture. In spite of glucosuria and phosphaturia the blood glucose concentration is not elevated and the serum phosphorus level is usually normal or low, but it may rise terminally with renal glomerular failure. In spite of systemic acidosis the urine is relatively alkaline and has a high content of ammonium. A generalized aminoaciduria usually involving excessive excretion of 10 or more amino acids, is found [18]. The high cystine excretion is just one facet of the more generalized aminoaciduria and need not occur even in the presence of large losses of other amino acids. This urinary amino acid pattern is therefore quite distinct as are the other clinical features from that of 'cystinuria,' in which excessive urinary losses of only cystine, lysine, arginine and ornithine are found (cf Chap. 42).

The familial incidence of the condition has been noted and will be examined below. The prognosis has generally been poor. Few patients survive more than a few years but deLam [19] recently reported 'a case of the syndrome' where the patient after having presented grave manifestations in infancy nevertheless reached adult age and may be looked upon as having made a complete recovery. Death is usually due to progressive renal failure or intercurrent infection. Post mortem examination sometimes reveals crystals of cystine extensively deposited throughout the body and cystinosis may be detected during life by examination of the conjunctiva and cornea with a slit lamp [20, 21] and of the bone

marrow [21-22] As indicated above controversy continues regarding the relationship of cystine storage disease to this *syndrome* Cystine storage definitely does not occur in all instances In 11 recorded autopsies on patients with glycosuria and rickets collected by McCune et al [15] cystine deposits were recognized in 4 and their presence was suspected in a fifth Because cystine crystals may easily be lost in fixation of tissues or may be unrecognized, it is impossible to ascertain the exact incidence of cystine storage at present It seems highly likely that the *syndrome* may occur in the absence of cystinosis in fact, cystinosis has not yet been described in the adult cases } ✓

In the adult the *syndrome* has the same features as in the infant, i.e., osteomalacia with multiple fractures and pseudofractures hypophosphatemia with hyperphosphaturia usually elevated alkaline phosphatase level generalized aminoaciduria with normal concentrations of amino acids in the serum and renal glucosuria Frequently there are albuminuria alkaline urine and mild or severe systemic acidosis occasionally hypokalemia and hypouricemia are present Crippling bone disease with pain and deformities dominates the clinical picture Glomerular filtration rate may be normal or only moderately reduced but the usual cause of death is glomerular insufficiency with retention of nitrogenous waste products and uremia

## PATHOPHYSIOLOGY

### CALCIUM AND PHOSPHORUS METABOLISM

Rickets or osteomalacia the most prominent clinical feature of the *syndrome* early focused attention on the derangement of calcium and phosphorus balance

A reduced serum phosphorus concentration has been a common finding although with terminal glomerular failure the level rises to normal or higher The serum calcium levels are usually normal while the alkaline phosphatase concentration is elevated The chemical features of osteomalacia [23] are therefore present in the blood and the bone disease has been repeatedly shown both radiologically and histologically to be rickets in the growing child and osteomalacia in the adult

Hyperphosphaturia has been considered an important feature of this condition [15-24-27] In addition elevated renal clearances of phosphate have been reported [28-30] indicating simply that renal excretion of phosphate remains relatively high despite the low serum phosphate concentration From the available evidence the usual causes of an increased renal clearance of phosphate do not seem to explain the findings in the *syndrome* The usual causes include primary and secondary hyperparathyroidism

Primary hyperparathyroidism seems definitely excluded by the absence

of hypercalcemia [17] and the presence of osteomalacia rather than osteitis fibrosa cystica. The reported findings in the parathyroid glands at necropsy are difficult to interpret but tumors have not been described [18], and mention of normal parathyroids has been made [9 10 31]. Surgical biopsy specimens of parathyroid glands from two of Hunter's cases were interpreted as normal [32].

Secondary hyperparathyroidism is not so readily excluded, histologically its presence or absence may be very difficult to ascertain. By definition this condition refers to a compensatory state requiring persistence of some *physiologic* stimulus to increase parathyroid activity above that normally required to meet the needs of the body. Albright and Reifenstein have considered a reduction in the concentration of serum calcium to constitute the effective stimulus [23], resulting in the following sequence of events: decreased serum calcium concentration  $\longrightarrow$  increased parathyroid activity  $\longrightarrow$  increased renal clearance of phosphate  $\longrightarrow$  reduced serum phosphate  $\longrightarrow$  return of serum calcium concentration toward normal.<sup>1</sup> To establish the presence of secondary hyperparathyroidism it is necessary to demonstrate a cause for a reduced serum calcium level. The usual cause is either an increased calcium excretion by the kidneys or a reduced absorption of calcium from the gut. However no such impairment in calcium metabolism can be traced in all cases of the *syndrome*.

Primary hypercalcaemia has not been noted and values for calcium excretion within a low or normal range are reported [15 28 30]. Furthermore the clinical picture bears no resemblance to that of idiopathic hypercalcaemia in which renal stone formation is the cardinal finding [33]. Hypercalcaemia is also a feature of renal tubular acidosis [23] and some patients with the *syndrome* have definite hyperchloremic acidosis [15 29] but others have severe osteomalacia with increased phosphate clearance in the complete absence of systemic acidosis [28 31 34] and with normal ability to excrete an acid load [25]. Albright and Reifenstein [23] postulated that the increased anion excretion imposed by the aminoaciduria would overtax the hydrogen ion excretory mechanisms (fixed cation-sparing mechanisms) of the kidney so that an increased calcium excretion would result causing demineralization of bone. This was an *a priori* assumption made admittedly without any cases to study. This view is disproved by the evidence that the amino acid excretion in the *syndrome* usually contributes only a small additional load of acids to those requiring excretion that severe bone disease may be present when aminoaciduria is a minimal feature [28 34] and that the renal acidifying

<sup>1</sup> The secondary hyperparathyroidism seen in chronic azotemic renal failure need not be considered here since an elevated rather than reduced serum phosphorus level is present. The renal clearance of phosphate is decreased not increased and the resulting osteodystrophy is not rickets or osteomalacia [23].

mechanisms of the kidney are not always overtaxed as evidenced by a normal response to further loading with strong acids [28]. Thus excessive renal loss of calcium can be excluded as initiating the disturbance in calcium and phosphorus metabolism.

An impaired absorption of calcium from the intestines is not a constant finding either. A lack of vitamin D in the usual doses is certainly not a factor. Treatment of the bone disease with usual or even moderately large doses of vitamin D provides inconspicuous or ephemeral benefit [10]. Normal blood levels of vitamin D [35-36] were reported in the case of Guild, Pierce and Iibenthal [24]. The normal levels of serum calcium together with the usually normal or even high levels of urinary calcium excretion [10] would seem to exclude simple failure of intestinal absorption of calcium. Not all investigators, however, agree on the last point. Bickel and associates [14] have interpreted their own results and those in the literature as indicating impaired intestinal absorption of calcium as the major cause of the bone disease. Recently Davies et al [37] have likewise reported a disturbance in intestinal absorption of calcium and phosphorus together with a very low urinary calcium excretion in a case of the syndrome.

Kyle and associates [28] have made important observations in an adult with the syndrome. By means of balance studies they showed (Fig. 37-1) the following: (1) Without therapy and while on a diet containing 1,200 mg calcium and 1,900 mg phosphorus daily, the patient was in negative calcium and phosphorus balance. The urinary phosphorus level was high, but there were normal amounts of calcium in the urine. Serum calcium concentration was normal and serum phosphorus low. (2) Alkali therapy with Shohl's solution (sodium citrate-citric acid mixture) had no effect on the calcium or phosphorus balance, a finding consistent with the complete absence of acidosis in their case. (3) 100,000 units of vitamin D daily improved calcium balance by decreasing fecal calcium loss, but urinary calcium excretion was not reduced and urinary phosphate excretion rose still higher. (4) Supplementary intake of neutral phosphate resulted in a positive phosphate balance with increased serum phosphorus concentration, although excretion of phosphate in the urine rose and most significantly there was a decrease in urinary calcium excretion. In this one case they were therefore able to exclude acidosis, vitamin D deficiency and an intestinal absorption defect as the cause of osteomalacia. Suggestive evidence was further presented that the hyperphosphaturia was not secondary to hyperparathyroidism, since the patient responded normally to the intravenous administration of parathyroid hormone with a further large phosphate diuresis. This would not be expected had excessive circulating parathyroid hormone initially been responsible for the hyperphosphaturia. These metabolic studies thus speak in favor of a primary defect in renal

tubular reabsorption of phosphate, a view suggested repeatedly in the past [10, 12, 27, 31, 35]

The absence of any apparent secondary cause for the increased renal clearance of phosphate had led most workers to postulate a primary defect in the renal tubular conservation of phosphate. It should be

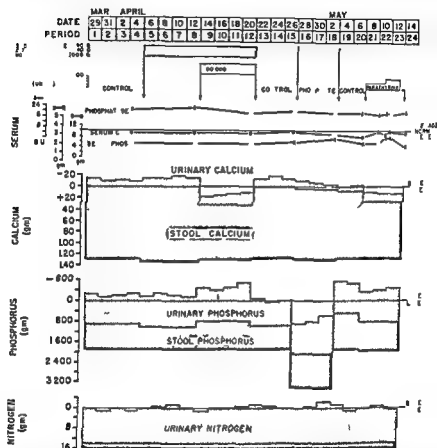


Fig 37-1 Results of the balance study showing calcium phosphate and nitrogen changes in a 42-year-old male with the syndrome (Data of Kyle Meroney and Freeman [28] reproduced by permission of the Journal of Clinical Endocrinology and Metabolism)

pointed out however that in the case carefully studied by Milne, Stanbury and Thomson [29] there occurred an apparent decrease in the high renal clearance of phosphate consequent to successful therapy of the bone disease with vitamin D and alkali. This finding suggests that the high renal clearance initially present in their case was a secondary rather than a primary defect. Correction of acidosis together with improvement of gastrointestinal absorption with vitamin D makes it difficult to evaluate the single clearance they report after therapy since



correction of acidosis alone might have reduced the phosphate clearance. The studies presented by Anderson, Miller and Kenny [34] also showed a drop in phosphate clearance when therapy with alkali and vitamin D improved calcium and phosphorus balances in their case. Thus with an increase in serum phosphorus concentration from about 2.0 to 3.5 mg per 100 ml the urine phosphorus rose only from 1.7 to 1.9 gm per 24 hr. Salas, Powers, Ulrich and Hayles [39] have confirmed this finding in two patients in whom balance studies were done. Whereas alkali administration with correction of acidosis failed to correct the hypophosphatemia or consistently alter the balances of calcium or phosphorus, administration of vitamin D in doses of 400,000 units daily for 30 days produced marked improvement in both patients. As a result of a marked decrease in fecal phosphorus and only a slight increase in urinary phosphorus during administration of vitamin D the phosphorus balance became positive and the renal clearance of phosphate dropped 37 and 44 per cent, respectively in their two cases.

These changes in renal phosphate clearance with repletion of body calcium and phosphorus stores are those classically ascribed to relief of secondary hyperparathyroidism [23]. However, an initial cause for secondary hyperparathyroidism can not be elicited in all cases of the *syndrome*. The alternate postulate that the phosphate loss is due to a primary defect in the renal tubular conservation of phosphate must still be retained. This defect perhaps may be specifically corrected by vitamin D in very large doses and indeed large doses of vitamin D may also have a favorable influence on other associated tubular defects (see below). Such a renal tubular defect might be modified in individual cases, however, by the existence of other factors known to affect calcium and phosphorus metabolism.

Davies et al. [37] make the interesting suggestion that a disturbance in intestinal as well as in renal tubular reabsorption may exist. The high intestinal phosphate would then secondarily interfere with intestinal absorption of calcium to explain the finding of a low urinary calcium excretion in some cases of the *syndrome*. Vitamin D, according to this view, corrects the intestinal as well as the renal defect in phosphate absorption, thereby improving the calcium balance together with the phosphorus balance. The secondary hyperparathyroidism induced by impaired intestinal absorption of calcium would thus be relieved.

#### ACIDOSIS AND ASSOCIATED ELECTROLYTE DISTURBANCES

Although not a cardinal feature of the *syndrome* as defined above, acidosis is a common finding in both the infantile and adult types. In untreated cases the acidosis may be severe. The serum  $\text{CO}_2$  content may fall as low as 9 mEq per liter [14]. The disturbances in acid-base balance have been carefully studied by Linder, Bull and Grayce [38] in an adolescent

by Milne and associates [29] Kyle and associates [28] and Sirota and Hammerman [30] in adult cases and by Bickel and associates [14] in several cases of the infant and childhood form.

The earliest thorough investigation into the acid base metabolism in the syndrome was that of Linder Bull, and Grayce [35]. Studying a 13-year-old girl they found the following: (1) The serum was of normal reaction but with slightly reduced bicarbonate concentration ( $c$  20 mEq per liter). (2) The rate of organic acid excretion was two to three times normal. (3) Following administration for 4 days of 2.5 mLq hydrochloric acid per kilogram of body weight per day, chloride excretion rapidly increased but ammonium excretion was distinctly subnormal, and titratable acids did not increase as expected. The excretion of the administered chloride was therefore, accompanied by a large loss of fixed cations.

The patient of Milne, when untreated, showed the typical picture of hyperchloremic acidosis. The serum bicarbonate was 14 mEq per liter (normal 20 to 30), the serum chloride was 118 mEq per liter (normal, 97 to 103) and the blood pH 7.1 to 7.3 (normal 7.35 to 7.43). The glomerular filtration rate as indicated by inulin clearance was reduced to 31 ml per min, a value approximately one-third to one-quarter the expected normal value. The plasma urea concentration was not elevated. An abnormal loss of bicarbonate in the urine was demonstrated. Even with the serum bicarbonate concentration reduced to 14 mEq per liter the daily excretion of bicarbonate in the urine was 20 mEq. Since at this plasma level all bicarbonate filtered is normally reabsorbed [40] the finding constitutes definite evidence for a reduced renal threshold for bicarbonate. Such bicarbonate wastage is a known feature of renal tubular acidosis [41]. It was not associated with an absolute loss of reabsorptive capacity for bicarbonate reabsorption was found to increase as in the normal person when the serum bicarbonate excretions rose with alkalinizing therapy.

The failure of the patient to excrete an acid urine was attributed to the presence of bicarbonate in the urine. Only after all the bicarbonate is reabsorbed from the tubular urine does the further excretion of hydrogen ions into the distal tubular urine succeed in making the urine strongly acid. When reabsorption of bicarbonate proximally is faulty so that bicarbonate is present in the distal tubular urine, hydrogen ion secretion by the distal tubule serves only to convert bicarbonate to carbonic acid. As the carbonic acid that is formed undergoes rapid decomposition to volatile carbon dioxide and neutral water the urine is not rendered acid.

Although Milne and associates found the rate of ammonium excretion in the urine to be normal or high for the pH of the urine, ammonium production was nonetheless totally inadequate in a biologic sense for the degree of systemic acidosis. The finding of an ammonium concentra-

tion relatively high for the urine pH would at present be ascribed to the concurrent potassium deficiency [47]

The disturbance in acid base regulation noted by Sirota and Hamerman [50] and their studies of this disturbance in their case were very similar to the observations of Milne and associates [9]. Their patient also had a hyperchloremic acidosis with urinary wasting of bicarbonate and impaired ammonium production.

All 14 of the children with the *syndrome* studied by Bickel and associates [14] were acidotic as indicated by a lowered  $\text{CO}_2$  combining power of the plasma. In each the degree of acidosis varied considerably quite apart from treatment and normal or only slightly lowered  $\bar{\text{CO}}_2$  values were found occasionally in almost all. Several of their patients were found to excrete large quantities of bicarbonate in the urine despite acidosis with reduced plasma  $\text{CO}_2$ . Defective ammonium formation and titratable acid secretion in some of the children likewise indicated either an inadequate or completely absent response to the administration of acid. Organic acid excretion was increased.

The findings of the above workers thus indicate a complex disturbance in renal function as the cause of the acidosis with defective tubular reabsorption of bicarbonate playing the major role and defective ammonium production, reduced titratable acidity and increased excretion of organic acids all contributing. As evidence of normal renal responsiveness to acidosis in this *syndrome* a patient of Fanconi [10] and one of McCune [15] are often cited to show that urine pH may be low while ammonia production and titratable acidity are high. In McCune's patient the plasma bicarbonate which was between 10.9 and 18.7 mEq per liter indicated severe acidosis. With such reductions of serum bicarbonate one might expect that even with a diminished tubular reabsorptive capacity for bicarbonate all of the decreased amounts of bicarbonate filtered would be reabsorbed thereby permitting production of an acid urine with increased ammonium and titratable acids. Thus Bickel et al. [14] noted that in one of their patients the bicarbonate loss stopped completely during a period of acid administration. At the same time the urine pH dropped from 14 to 3.8 while the plasma  $\text{CO}_2$  fell to the alarmingly low level of 7.3 mEq/l. At the present time it may be justifiably concluded that even when the renal hydrogen-excreting mechanisms appear to be functioning normally the response is totally inadequate for the degree of systemic acidosis. If recognition is given to the expected absence of bicarbonate in the urine when the patient is severely acidotic it seems that the renal disturbances together with the moderate increase in organic acid excretion mentioned adequately account for the acidosis.

Ketonaemia has been a common finding in this *syndrome*. It is not surprising in view of the renal glucosuria and inanition but has not been

proved in any instance to be of quantitative importance in producing acidosis

The impression should not be left that acidosis or impaired renal mechanisms for hydrogen ion secretion are essential parts of the disease process. In the well-documented case of Kyle et al [28] the patient had a normal plasma bicarbonate and in response to an acid load of 130 mEq ammonium chloride daily for 3 days produced as acid a urine with as high a titratable acidity as did a normal control similarly treated and an even higher ammonium content than the control. The bone disease and other features of the *syndrome* were nevertheless all present. The variability in the number and severity of the features apparent in any given case will be considered further below.

A deficiency of potassium which is causally related to the defect in hydrogen ion excretion has been generally appreciated since the study of Milne et al [29] although Linder and associates previously [38] commented on the loss of sodium and potassium in association with the production of dehydration and symptoms of potassium deficiency. Case histories of this *syndrome* refer frequently to unexplained bouts of weakness, paralysis and collapse. An interesting occurrence termed "dextrose shock" has been noticed. This consists of severe and sudden peripheral collapse during sugar tolerance tests. Both Debré [8] and Fanconi [10] witnessed such episodes in conjunction with the oral administration of dextrose for glucose tolerance tests. Debré's patient became restless and pallid and then lapsed into fatal coma. In neither of the two patients of Fanconi did the episode prove fatal. These episodes are most probably the result of hypokalemia, a finding repeatedly noted in this *syndrome* [14, 17, 29]. In addition to the weakness, paralysis and collapse, potassium depletion is undoubtedly responsible for at least two other common findings, polyuria and, at post mortem, vacuolar changes affecting the renal tubular epithelium. Polyuria with isosthenuria had been frequently noted and in some instances shown to be resistant to antidiuretic hormone [38]. This is characteristic of the nephropathy of potassium deficiency [42]. Recently Davies et al [37] have demonstrated improvement in urinary acidification following correction of potassium deficiency in their patient. They considered the potassium depletion to be primary rather than secondary to the disturbance in acid base regulation and hence to represent a separate tubular reabsorptive defect.

#### GLUCOSURIA

Glycosuria was recognized early in the delineation of this *syndrome* and has remained one of its most definite and least controversial features. The reducing substance in the urine was found to be glucose by Fanconi [9, 10] and subsequently by others. It is of renal origin, i.e. the result of a lowered renal threshold for glucose. In the few instances in which diabetic

like glucose tolerance test results have been observed preceding carbohydrate depletion was probably responsible. In many instances cases have been incorrectly diagnosed and treated with insulin. The glucosuria is variable, ranging from mere traces to 4 to 5 gm per 100 ml [14-15]. The elimination of sugar is characteristically continuous at least in the more severe forms of the syndrome but it may be intermittent and not infrequently will disappear terminally.

The presence of glucosuria has had a special significance in this syndrome in helping to localize the site in the nephron where the functional impairment resides. The findings of Richards [43] are generally accepted as demonstrating that glucose reabsorption occurs in the proximal tubule. Hence the presence of renal glucosuria has constituted a strong argument in favor of placing the disturbance in the proximal tubule. The location of the disturbance in the kidney will be considered in greater detail below.

### AMINOACIDURIA

Aminoaciduria is the most recently recognized feature of the syndrome. Although suspected by Fanconi [10] it was not proved until the work of McCune and associates [15]. It has been investigated in detail only since the techniques of chromatography were applied to the problem notably by Dent [18] who since 1947 in a series of studies [18-44-46] has characterized the main features of the aminoaciduria.

The normal individual excretes some 200 to 400 mg amino acids daily [47]. Amino nitrogen constitutes less than 3 per cent of the total urinary nitrogen [18]. Figure 37-2A and B show the urinary pattern of amino acids from a normal infant and adult respectively. The trace amounts of amino acids in normal subjects are to be contrasted with the high excretion rates shown by a child and adult with the syndrome (Fig 37-3A and B respectively). These figures illustrate the main features of the aminoaciduria: (1) It is a generalized aminoaciduria involving usually 10 or more amino acids. (2) The pattern of amino acid excretion is variable from case to case but seems to remain quite constant with time in any individual case [46]. (3) Cystine excretion may be increased or normal even in the presence of cystinosis. Renal calculi of cystine have so far been reported in only one case [48]. These features distinguish the aminoaciduria sharply from that seen in cystinuria in which the excretion of only four amino acids is increased and these are always the same (cystine, lysine, arginine and ornithine) [49-51] as shown in Fig 37-4.

The generalized aminoaciduria is reminiscent of the overflow type seen in severe liver disease [52-53]. However, Dent has demonstrated that the plasma amino acid concentrations are normal or low [18] rather than elevated as in liver disease. Harper and associates [54] have also reported essentially normal plasma levels for several amino acids that were present

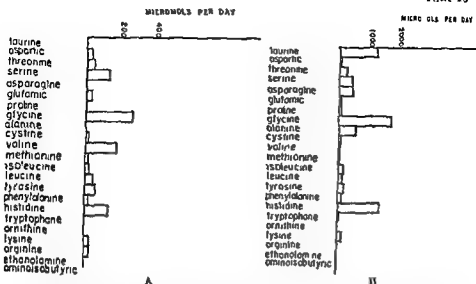


Fig 37.2 A The daily amino acid excretion in the urine of a normal infant (according to Jonxis [73]) B The daily amino acid excretion in the urine of a normal adult (Data of Harrison and Harrison [83] reproduced by permission of the Journal of the American Medical Association)

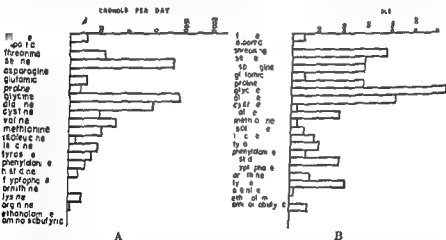


Fig 37.3 A The daily amino acid excretion in the urine of a child with cystine storage disease and the syndrome (From Harrison and Harrison [83]) B The daily amino acid excretion in the urine of an adult with the syndrome (Data of Evered [47] recalculated by Harrison and Harrison [83] and reproduced by permission of the Journal of the American Medical Association)

in excess in the urine. Although Dent's findings were contested by Bickel and Smellie [21] who reported levels 50 to 100 per cent above the normal range. Evered has recently [47] reexamined the problem with the more precise method of quantitative ion-exchange column chromatography and has convincingly supported Dent's results. Thus the renal clearance

of amino acids: high in the *syndrome* and a defect in renal tubular reabsorption would seem to be responsible for this feature.

Because knowledge regarding the normal tubular mechanisms for amino acid transport is only fragmentary the defect responsible for the aminoaciduria in the *syndrome* is not understood. Latham, Baker and Bradley [55] found a gross aminoaciduria during fastings in their patient with the *syndrome*. However during an intravenous infusion of amino acids (hydrolyzate of fibrin) the increase in amino acids in the urine was not in excess of that occurring normally during a similar infusion. They suggested that the defect in tubular reabsorption of amino acids may involve only a small percentage of the nephron population. This conclusion is at variance with both the reported widespread distribution of the morphologic changes in the kidney (see below) and the results of Robson and Rose [56] who found a high initial clearance of cystine in their patient which rose only slightly following infusion of lysine to equal the clearance of inulin. Beyer and associates [57] have studied competition for reabsorption of various amino acids by the renal tubules and indicated that at least three separate transport systems are present in the dog for tubular reabsorption of different amino acids. If these studies in the dog are applicable to the human kidney patients with the *syndrome* lose amino acids from all three groups. Little [58] in studies also with the dog demonstrated that the reabsorptive processes of the tubules of amino acids are distinct from those involved in glucose absorption.

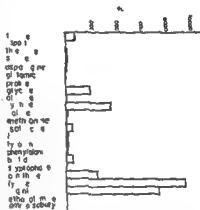


Fig. 34-4 Urinary excretion of amino acids by a patient with cystinuria (Data of Eierd [54] recalculated by Harrison and Harrison [55] and reproduced by permission of the Journal of the American Medical Association.)

## MORPHOLOGIC CHANGES

Only recently has any lesion been described at necropsy which appears to have some specificity to this disorder. There are reasons to believe that a lesion in the proximal renal tubule could most easily account for the renal disturbances of the *syndrome*. Thus there has been great interest in the report in 1953 of Clay, Darmady and Hawkins [59] of a characteristic lesion affecting this portion of the nephron. By using a microdissection technique to isolate individual nephrons these workers ob-

served two apparently characteristic types of lesions present in the kidneys of two patients with the *syndrome* (1) The swan neck deformity illustrated in Fig 37 5B, was present 100 times out of 101 consecutive nephrons examined at random. In this lesion normal glomeruli are attached to a narrow neck in which the epithelium is of a low cuboidal form for a distance of 0.3 to 1.0 mm. Further down the epithelium suddenly expands into the height and shape of the normal proximal tubule (Fig 37 5A) but differs from it in possessing a greater translucency. (2) The second change is a shortening of the whole proximal convoluted



Fig 37-5 A Normal human glomerulus and proximal tubule as seen following microdissection. B Glomerulus and nephron from a patient with the *syndrome* showing the swan neck lesion affecting the proximal tubule. C Glomerulus and nephron from a patient with the *syndrome* showing the atrophy and shortening of the proximal tubule. (From Darmady and Stranack [60] reproduced by permission of the British Medical Bulletin.)

tubule including its narrow portion. The authors considered that the proximal tubules were atrophic and about one-fourth to one-half the normal length. This lesion is also shown in Fig 37 5C in which a normal nephron has been included for comparison.

More recently Darmady and Stranack [60] have reported their further experience with the microdissection technique in 11 cases<sup>21</sup> where the clinical history conformed strictly to that laid down for Lignac Fanconi disease (cystinosis).

In 8 the proximal tubule was shorter than normal and its first portion was replaced by a thin and narrow neck for about one-third of its length, i.e. the swan neck defect. The remaining 3 cases showed a hypotrophic lesion throughout the length of the proximal tubule. In the recent report two additional patients are mentioned who in life had glycosuria, phosphaturia, hypopotassemia, abnormal amino aciduria, and dwarfism but who lacked cystinosis. Both these patients showed the typical swan neck lesion. It is of special interest that one of these two patients, aged 46, had the adult form of the *syndrome* while



the remaining 12 were all of the infantile or childhood form, the oldest being 9 years of age

The absence of any associated regenerative changes in the kidneys together with the regularity and frequency of the proximal tubular lesions led the authors to the conclusion that these represented congenital defects even though the clinical syndrome often did not become manifest for some time after birth. The lesions seemed specific. They were not found in tissue obtained from a case of renal amyloidosis and were distinguished from a somewhat similar lesion described by Oliver [61] in chronic nephritis in which only a small fraction of the nephrons are affected.

Other renal lesions have been described in this *syndrome* which do not seem to be specific. Vacuolation of the tubular epithelium has been noted [16] but this is attributable to the associated potassium depletion which is often encountered in these patients and which is known to produce similar vacuolar changes [41]. Atrophic changes in the distal tubular epithelium similar to those seen in acute tubular necrosis have also been reported [59] as have varying degrees of renal fibrosis. Since progressive renal impairment with uremia is a common outcome of this *syndrome* fibrosis and loss of nephrons are perhaps to be expected at necropsy but their relationship to the structural basis of the observed renal dysfunction is difficult to ascertain. Pyelonephritis may be responsible for the progressive renal failure. Potassium depletion may in turn predispose to pyelonephritis [62] although this suggestion requires further support [42].

In addition to renal pathologic changes changes in other tissues have also been noted. Skeletal changes consistent with rickets or osteomalacia occur as expected [6]. Fatty liver and focal necrosis of the liver or cirrhosis have been noted repeatedly though not invariably. Their relationship to the remainder of the *syndrome* remains obscure [15-27]. It has been suggested that the urinary amino acid loss might be the cause of the cirrhosis [27] but evidence is lacking that such loss is of sufficient magnitude to be considered seriously as a cause of protein depletion. Cystine crystals have been reported in the tissues of many but certainly not in all the autopsy reports. The fact that Bickel and Smellie [21] were able to demonstrate cystine crystals *ante mortem* in bone marrow biopsies from all 17 of their cases suggests that a more careful search will reveal crystals in a larger fraction of cases.

## ETIOLOGY AND GENETICS

Fanconi is generally credited with the view that impairment of the reabsorptive capacity of the renal tubules without a corresponding reduction in glomerular filtration volume could account for the major features

of the *syndrome*. A tubular reabsorptive defect seems to be directly responsible for the aminoaciduria, glucosuria, phosphaturia and the tendency to excrete an alkaline urine, and secondarily responsible for the systemic acidosis and skeletal changes. The anatomic changes found by Darmady and associates [59-60] appear to provide a morphologic basis in the proximal tubule for such a defect. The proximal tubule is thought to be the site of reabsorption of glucose [43] and Fanconi [63] considers the proximal tubule to be the site of reabsorption of amino acids and phosphate as well. Hence the morphologic changes are to this extent consistent with the functional derangements.

When one asks the important next question, namely, what is the cause of the renal tubular defect, a unitarian approach to the *syndrome* is no longer tenable. The familial occurrence of this rare *syndrome* early suggested an hereditary basis for the disease. Recently, however, the renal defects associated with the *syndrome* have been recognized repeatedly under circumstances in which renal damage was clearly acquired. Harrison and Harrison [64] reported that injections of maleic acid into rats may produce renal glucosuria, phosphaturia and a generalized aminoaciduria. It had previously been demonstrated that this compound interfered with the renal excretion of acid by acidotic dogs [65]. These renal disturbances proved reversible. Spencer and Langlen [66] have reported gross glucosuria and aminoaciduria without elevated blood glucose or amino acid concentrations occurring transiently during the recovery phase of acute renal failure in a patient who suffered an extensive Lyol burn. Experimental uranium poisoning may produce glucosuria and aminoaciduria [67-68]. Gross renal aminoaciduria has been observed in lead poisoning [69-71] and in cadmium, mercury and uranium poisoning [72]. In at least one instance of lead poisoning in a child [70] the aminoaciduria was associated with glycosuria, persistent hypophosphatemia and rickets. The acquired *syndrome* in this child resolved after treatment of the lead poisoning. Vitamin D deficiency has been associated with renal aminoaciduria [73]. Of considerable interest is the association of multiple myeloma and the adult form of the *syndrome* reported recently by Engle and Wallis [74] and noted earlier by Sirota and Hamerman [30]. These clinical and experimental observations indicate that the *syndrome* may have multiple etiologies in which the common denominator is renal tubular injury with impairment especially of the systems that are important in the renal tubular handling of amino acids, glucose and phosphate.

The hereditary nature of this *syndrome* was first noted by Abderhalden [1] and has been referred to repeatedly since. Duplication of the *syndrome* in siblings and blood relationship between parents have been noted [15]. Dent and Harris [75] concluded from their study of the genetics of this *syndrome* that 'the simplest view is that we are concerned with a simple

recessive character. The difficulty of characterizing genetically transmitted traits in human disease as either simply dominant or recessive has been recently emphasized [76]. What is definite however is that this rare syndrome may affect more than one sibling and that there is a high instance of parental consanguinity [15-75]. Clarification of the genetic mode of transmission is made difficult because acquired renal tubular damage can mimic this syndrome. There also remains uncertainty as to whether individuals who develop the adult form of the syndrome have had a renal tubular defect since birth or whether the lesion itself developed only in adult life and accordingly whether the infantile and adult forms are the same disease. Dent and Harris [75] described an interesting family in which three adult siblings had biochemical abnormalities closely similar to those found in the propoitus with the adult form of the syndrome. Each sibling resembled the patient in having generalized aminoaciduria, mild acidosis and hypophosphatemia but unlike their afflicted sister they were clinically well with no evidence of bone disease. How long the 3 siblings had had these biochemical disturbances and whether they might have developed clinically apparent disease with time remained unanswerable. The biochemical defects could conceivably be present from birth even in the adult cases but not clinically manifest unless the patient was confronted by environmental factors e.g. pregnancy and lactation which would place a strain on the impaired mechanism for calcium and phosphorus conservation. On the other hand adult cases have been reported in which a retrospective search for glycosuria on preceding urinalyses have yielded negative results [76]. Even in the infantile form Bickel has reported well-documented cases indicating that the abnormality in renal function was not manifest during the first few months after birth [77].

Dent has pointed out [45] that in the hereditary diseases generally which include some forms of aminoaciduria all the cells of the body carry the defective gene while only in the kidney in the syndrome does the abnormality resulting from the defective gene give rise to clinically recognizable disturbances. In this regard the association of cystine storage with the syndrome in some instances remains a confusing feature. Most workers accept the results of Dent [18] and of Levered [47] referred to above which indicate no elevation of plasma amino acid levels in the syndrome although Bickel claims that elevated plasma levels may be present [14-77]. If elevated plasma amino acid levels are not present some local factor which is not essential to the development of the syndrome must be responsible for the widespread deposition of cystine crystals seen in patients with cystinosis. DeToni's view [13] that cystinosis and the syndrome are different entities whose coexistence is the result of an acquired renal tubular lesion producing the syndrome as a complication of cystine storage may have merit. This view has also been stated by

of the *syndrome*. A tubular reabsorptive defect seems to be directly responsible for the aminoaciduria, glucosuria, phosphaturia, and the tendency to excrete an alkaline urine, and secondarily responsible for the systemic acidosis and skeletal changes. The anatomic changes found by Darmady and associates [59, 60] appear to provide a morphologic basis in the proximal tubule for such a defect. The proximal tubule is thought to be the site of reabsorption of glucose [43], and Fanconi [63] considers the proximal tubule to be the site of reabsorption of amino acids and phosphate as well. Hence the morphologic changes are to this extent consistent with the functional derangements.

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*drome*) To this list should probably be added impaired tubular reabsorption of bicarbonate as seen in renal tubular acidosis [41] which frequently occurs in the *syndrome* as discussed above Dent [44] had earlier proposed a somewhat similar subdivision of possible conditions arising from combinations of separate tubular reabsorptive defects Thus it is clear that the *syndrome* constitutes no clear-cut entity with distinct clinical functional or etiologic boundaries but merges into the spectrum of disturbances arising from all possible renal tubular defects

## TREATMENT

The problem of management of a condition whose clinical features are the result of renal losses of certain body constituents would seem to be one primarily of replacement therapy The renal losses of glucose or of amino acids in this condition have not constituted a problem because they are quantitatively not sufficient to produce nutritional disturbances Acidosis and phosphate loss require treatment in order to heal the skeletal lesions The rickets and osteomalacia have repeatedly been found to be resistant to usual amounts of vitamin D but improvement in skeletal and biochemical disturbances occurs with large doses of vitamin D (50 000 to 400 000 I U daily) [21 38 39 44 82] It is of special interest that the aminoaciduria may be diminished although not necessarily restored to normal values by large doses of vitamin D [83]

In view of the excessive renal clearance of phosphate it would seem rational to supplement the dietary intake of phosphate and to replace the excessive loss Until the skeleton has remineralized supplementary calcium may also be needed but a high calcium intake together with large doses of vitamin D considerably increase the risk of hypercalcemia and should be undertaken only if frequent and careful follow up determinations of serum and urine calcium levels are possible

The acidosis has been treated successfully with the solution of Shohl [30 34] which contains 140 gm citric acid and 98 gm sodium citrate per liter Since potassium deficiency is commonly present potassium bicarbonate or citrate may be necessary However as chronic acidosis is presumably the cause of the potassium depletion once acidosis has been relieved no further excessive potassium losses should be incurred and supplementary potassium should not be continuously required

Combinations of the above replacement therapy have increased considerably the optimism with which immediate prognosis in this *syndrome* may be regarded Since therapy merely replaces losses rather than cures the tubular defect it is evident that therapy must be continued indefinitely Replacement therapy does not seem to prevent the slow progression of renal impairment with ultimate death from glomerular failure and uremia [8<sub>4</sub>] Since terminal renal failure may perhaps occur more

Harrison [78] No one has yet produced a satisfactory explanation of how a renal tubular defect resulting in loss of amino acids can simultaneously result in excessive deposition of cystine in the tissues. Clearly some factor in addition to the renal lesion must be present in these cases.

The findings of Salassa, Powers, Ulrich and Hayles [39], who carefully studied the effects of large doses of vitamin D (400 000 units daily) for a period of 30 days in two patients, deserve special mention. Not only did the balances of calcium and phosphorus improve markedly with a reduction in renal clearance of phosphate, but all other parameters that were studied likewise improved. Thus the aminoaciduria decreased, glucosuria diminished, serum bicarbonate concentrations rose to normal, and serum chloride levels fell to normal. The decrease in aminoaciduria occurred in the absence of a detectable change in the plasma concentration of amino acids. Curiously, the acidosis corrected itself in spite of a considerable decrease in urinary ammonium excretion. Presumably a more acid urine with a high titratable acid excretion must have resulted from the vitamin D, but in the one case where titratable acid excretion rates were reported, no increase was found. These interesting results require confirmation in a larger number of cases. The fact that vitamin D deficiency is associated with an aminoaciduria and that the aminoaciduria is corrected by administration of vitamin D [73] supports at least some of the findings of Salassa and associates. Perhaps some processes in the renal tubule which normally are dependent upon vitamin D are defective but in some cases at least are partially correctable by huge doses of vitamin D. Much more work will be required before knowledge of the normal tubular reabsorptive mechanisms and their relationship to the action of vitamin D will permit more than conjectures on the etiology of this condition.

The arbitrary status of this syndrome has been emphasized by recent reports in which all possible combinations of the glucosuria, phosphaturia and aminoaciduria have been observed. Thus Luder and Sheldon [79] reported "A Familial Tubular Absorption Defect of Glucose and Amino Acids" in which only glucose and amino acid reabsorption were impaired. This defect appeared to be the result of a dominant genetic characteristic. They considered this defect to be one of seven possible combinations of defects deriving from abnormalities of tubular reabsorption of phosphate, glucose and amino acids: (1) glucose alone (as in renal glucosuria); (2) phosphate alone (possibly vitamin D-resistant hypophosphatemic rickets [44]); (3) amino acids alone (cystinuria or hepatolenticular degeneration [80-81]); (4) glucose with phosphate (combination of renal glucosuria and hypophosphatemic rickets [44]); (5) phosphate and amino acids (aminoaciduria in vitamin D deficiency [73]); (6) glucose and amino acids [79]; (7) phosphate, glucose and amino acids (the syn-

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readily in the potassium-deficient subject [62], any evidence of urinary tract infection should be carefully investigated and specifically treated.

In contrast to 'cystinuria, cystine excretion in the urine may be either normal or only moderately increased. Nephrolithiasis due to cystinuria in this *syndrome* has been reported so far on only one occasion [48] and no specific measures are ordinarily required for its prevention.

Those who claim cures for the *syndrome* must take cognizance of the acquired forms secondary to reversible renal tubular damage from a wide variety of agents.

## SUMMARY

1 A *syndrome* characterized by rickets or osteomalacia with renal glucosuria, aminoaciduria, phosphaturia and frequently acidosis has been reviewed. This *syndrome* may appear early in life (infantile form) or manifest itself first during later life (adult form).

■ The associated physiologic disturbances are best explained as consequences of a defective renal tubular reabsorption of glucose, amino acids, phosphate and bicarbonate.

3 A characteristic morphologic change has been described in the proximal tubule of these subjects which provides a plausible anatomic basis for the functional disturbances.

4 The clinical and experimental observations which have been reviewed indicate that the *syndrome* may have multiple etiologies in which the common denominator is injury to the proximal renal tubule with impairment of its normal reabsorptive functions. Thus acquired forms of the *syndrome* are now recognizable in addition to the genetically determined forms which were initially described and in which the hereditary factor acts like a simple recessive character.

5 Rational therapy aimed at replacing abnormal renal losses is outlined. Reversal of the morphologic changes in the kidney once they have developed seems unlikely and no way of halting the gradual development of glomerular failure and uremia is known at present.

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reached levels several fold greater than the concentration in glomerular filtrate at  $T_m$ . The display in the glucose titration curve for the whole kidney as well as for the individual nephron could be explained on this basis.

The nature of the hypothetical cellular carrier if indeed one exists has not been elucidated. It has been postulated that the initial step in transport is phosphorylation of glucose mediated by hexokinase and adenosine triphosphate and subsequent dephosphorylation through the action of a phosphatase [16-17]. The glucose 6-phosphate formed in the first reaction is assumed not to diffuse out of the cell and the removal of free glucose by phosphorylation would then favor the inward movement of glucose in the direction of the concentration gradient. Hexokinase, non-specific alkaline phosphatase and the specific glucose 6-phosphatase are found in proximal tubular cells. However, the weight of evidence is heavily against a phosphorylated sugar as an intermediate in the transport process [10]. When phlorizin was administered to animals to produce almost complete block of glucose reabsorption, no inhibition of alkaline phosphatase in the proximal convoluted tubules, shown histochemically, could be found [18]. Phlorizin in doses which produced profound glycosuria did not alter the time curve of disappearance of injected  $P^{32}$ -labeled inorganic phosphate from the plasma of cats [19]. These data of Dratz and Handler [19] indicated that in the presence of phlorizin, normal or even accelerated synthesis of glucose esters is seen. The authors concluded that if a highly active fraction of either glucose 1-phosphate or glucose 6-phosphate mediates renal tubular transport of glucose, then this must be present in amounts too minute to be detected by their technique. In addition, there are two clinical observations which fail to support the unique role of either alkaline or glucose 6-phosphatase in the transport reactions. Cori noted the absence of glycosuria in patients with glycogen storage disease in whom the specific glucose 6-phosphatase was lacking [20]. Similarly, normal carbohydrate metabolism has been observed in subjects with hypophosphatasia in whom alkaline phosphatase activity is decreased or absent [21].

Additional evidence against the phosphorylation hypothesis has been obtained through studies of active transport of sugars in the small intestine, a situation analogous to that in the renal tubule. The experiments of Solis on the substrate specificity of intestinal hexokinase demonstrate clearly that this enzyme cannot directly participate in active transport [22]. He found that several hexoses actively transported by the intestine were not phosphorylated by intestinal homogenates. More directly, it has been shown that certain hexoses lacking a hydroxyl group at carbon atoms 1 or 2 can still be transported by intestinal sacs *in vitro* against a concentration gradient [23]. Further examination of the substrate specificity of this reaction has shown that these compounds are transported

transport of glucose. This subject has been recently reviewed by Taggart [10].

The application of micropuncture techniques has demonstrated in various Amphibia and mammals that glucose is present in the glomerular filtrate in the same concentration as in the plasma water [11, 12]. In the proximal convoluted tubule sugar is reabsorbed against an increasing concentration gradient, so that by the mid point of this tubule little glucose remains in the lumen. The reabsorptive process which is blocked by the glycoside phlorizin, is not restricted to any particular segment of the proximal tubule, but it is not demonstrable in the distal tubule. In the presence of phlorizin the concentration of reducing substance in the tubular urine may increase to as high as three times that of plasma as water is reabsorbed. In phlorizinized *Necturus*, glucose can diffuse back toward the plasma if the concentration in the tubular lumen is significantly greater than in plasma.

It has been shown in the dog by Shannon [13] and in man by Smith [14] that as the concentration of glucose in the blood is progressively increased the amount reabsorbed ( $T$ ) by the tubules approaches a maximum ( $T_m$ ). Shannon assumed that as the load of glucose presented to the tubules increases progressive saturation of the reabsorptive mechanism occurs until a maximum level is reached and the excess is excreted in the urine. He postulated that the sequence of reactions in glucose transfer involves (1) a reversible combination of the solute with a hypothetical cellular element present in constant but limiting amounts and (2) decomposition of this complex by a reaction which is first-order in type and rate limiting for the whole process.

Although Shannon's data fitted well with his hypothesis clearance data obtained in man show variable deviations, or splay, from the ideal curve [14]. The explanation has been offered that even in the normal kidney with loads less than the maximal average the transport in some individual nephrons will be less than in others [15]. Since the capacity of some tubules for reabsorption will be exceeded at relatively low levels glucose would be excreted from these nephrons before the average maximal load is reached. It has been further assumed that the titration curves for both kidneys would deviate from the "theoretical" in proportion to the degree of heterogeneity in the nephron population.

It has however been pointed out by Mudge [15] and Berlner [49] that the assumptions on which this explanation is based can be only an approximation, i.e., that each nephron excretes no glucose until completely saturated. There are no experimental data which conclusively prove this. On the contrary if Michaelis-Menten kinetics are applicable to the process of glucose transport across a tubule cell it is apparent that maximal rates would not be attained until concentration of the substrate

## NATURE OF THE TUBULAR ABNORMALITY IN RENAL GLYCOSURIA

It is not possible to localize the fundamental defect in renal glycosuria. With the exception of two cases published in 1935 and 1939 there is no recent report of pathologic anatomic findings in this disease. Marble [1] has pointed out that in the earlier publication of Grote and Heilmann [36] the patient probably had true diabetes mellitus. The other case reported by Monasterio [37] was evidently one of true renal glycosuria with a similar disease in a sibling and diabetes mellitus in one parent. Histologic examination of a biopsy specimen of one kidney showed in many places marked flattening of tubular epithelium; this was occasionally so striking that the epithelium resembled endothelium. The author was careful to note that the relationship of this change to the functional abnormality was not certain.

There have been several studies of patients with renal glycosuria using clearance techniques [38-42]. The glomerular filtration rate [38-40], secretion of  $\beta$  aminohippuric acid [38], reabsorption of phosphate [43] and amino acids [15] have been reported normal in these patients. There is no evidence of generalized progressive renal failure.

Data on glucose reabsorption as reported by several groups of investigators are summarized in Table 38-1. It was originally proposed by Reubi [38] and Bradley [40] that the patients seemed to fall within two separate groups: those with normal glucose  $T_m$  and those with low glucose  $T_m$ . Reubi concluded that in those patients with low glucose  $T_m$  a familial tendency was evident. Bradley thought the reverse was true. Taggart [44] in reviewing the available literature including the data collected by Bradley, Reubi, and Lambert, concluded that the cases did not appear to fall into two distinct categories according to the glucose  $T_m$ , but were evenly distributed from the lowest to the highest values. This is evident as shown in Table 38-1.

Data obtained with the titration method of Smith [14] are of considerable interest. Use of this technique in the patients with low glucose  $T_m$  has led to the conclusion that the defect in glucose reabsorption is diffuse involving most if not all nephrons. In the other individuals with renal glycosuria in whom the glucose  $T_m$  was within the normal range both Reubi and Bradley commented upon the exaggerated splay in the titration curves. They attributed this to a heterogeneity among nephrons whether on a functional or anatomic basis. Reubi further suggested that in these individuals there were actually two distinct groups of nephrons. However, as previously mentioned, configuration of the titration curve may be a function of the kinetics of the tubular transfer reactions rather than an expression of heterogeneity among nephrons. For example, a

which possess a  $\beta$  pyranose ring, a methyl or substituted methyl group at carbon 5 of this structure, and a hydroxyl group in the glucose configuration at carbon 2 [24] a structural specificity differing distinctly from that of mammalian hexokinase [25] Reactions which involve removal or transfer of oxygen at carbon 2 of the sugar molecule or which require the presence of carbon bound hydrogen at the same position have further been excluded [26] In the kidney it has also been shown by Chinard who infused glucose  $1\text{ C}^{14}$  into the renal artery of anesthetized dogs, that the sugar is transported without breakdown and resynthesis of the six carbon chain [27]

Some comment concerning the inhibitory effect on renal sugar transport of the glycoside phlorizin is pertinent since many of the hypotheses discussed above assume that the action of this compound is highly specific Phlorizin has been shown to inhibit several nucleotide requiring enzymes such as phosphorylase [28] Ehrlich ascites tumor hexokinase [29] mutarotase [30], and aerobic phosphorylation in homogenates of guinea pig kidney cortex [30] In addition, it inhibits the entrance of sugars into erythrocytes [31] and Ehrlich ascites tumor cells [32] which do not possess a mechanism for the accumulation of these substances against a concentration gradient It has recently been shown that slices of rabbit and rat kidney cortex can accumulate  $\beta$  galactose  $1\text{ C}^{14}$  a sugar reabsorbed by mammalian kidney tubules, against an apparent concentration gradient [33] In the absence of oxygen or in the presence of dinitro-ortho cresol (an inhibitor of oxidative phosphorylation) the amount of galactose entering the cells is limited to that expected from diffusion equilibrium The extent of accumulation in a given time period is a function of the external galactose concentration and shows a maximal rate Glucose competitively inhibits this accumulation Phlorizin in concentrations previously shown to be active *in vivo* inhibits the entrance of galactose into the tissue not only under conditions where accumulation can occur but also when accumulation has been inhibited by dinitro ortho cresol In the light of the results obtained with this *in vitro* model other experiments which minimize the role of diffusion of glucose from the tubular lumen in the presence of phlorizin must be reevaluated Diffusion becomes evident only when the process of active transport is blocked [34]

In summary glucose is present in the glomerular filtrate in concentration equal to that in the plasma water It is reabsorbed in the proximal convoluted tubules against an ever increasing concentration gradient Reabsorption of the sugar is mediated by energy requiring processes has a restricted specificity and is susceptible to inhibition by phlorizin, which in some manner restricts penetration of sugar into the cell A maximal rate of transport is observed both *in vitro* and *in vivo* The nature of the transport mechanism remains obscure and to date no chemical reaction involving the transported sugar has been demonstrated

of the tubular membrane towards the diffusion of glucose could also result in a change in the shape of the titration curve in the same direction.

One can therefore only speculate concerning the tubular abnormality in renal glycosuria. It is possible that no one explanation will suffice to account for the mechanism of the defect in all cases.

## GENETICS

The abnormality in renal glycosuria is probably inherited as a Mendelian dominant characteristic [2 7 8]. Further examination of members of affected families using more refined techniques of examination (such as Smith's titration method [9]) is indicated before this can be established with certainty.

The most complete study of the heredity of renal glycosuria was published in 1927 [47]. Hjarne was able to obtain information on 141 out of 199 individuals in three generations who had common ancestors in the eighteenth century. Glycosuria with normal blood sugar levels was found in 18 on unlimited carbohydrate intake and an additional 6 persons had glycosuria during the course of a glucose tolerance test only. Hjarne concluded that the defect was inherited as a dominant characteristic in view of the following:

- 1 Glycosuria occurred in male as well as female members of the family.
- 2 Where neither parent had glycosuria none of the offspring had the abnormality.
- 3 Where either of the parents had glycosuria some of the children generally had it.

Although true diabetes mellitus was present in 7 members of the family studied by Hjarne, the mode of inheritance of this defect was not similar to that of renal glycosuria. Similar conclusions regarding inheritance of renal glycosuria were reached by Schnell [7] and supported by the report of Brown and Poleshuck [48]. So far the heterozygote has not been differentiated from the homozygote—although I roesch et al [46] have suggested from the evaluation of titration curves in three brothers with the defect the possibility that the defect may be expressed in the heterozygote but more distinctly in the homozygote.

## SUMMARY

1 Renal glycosuria is an abnormality in which glucose is excreted in the urine at normal concentrations of blood glucose. The condition probably inherited as a Mendelian dominant trait is a benign one unassociated with any clinical abnormality.

2 It is still not certain whether several different abnormalities are included under the name *renal glycosuria*. Separate entities could depend

TABLE 38-1 DATA ON GLUCOSE REABSORPTION

| No                   | Case | Sex | Inulin clearance ml/min | Glucose $T_m$ mg/min | Ref  |
|----------------------|------|-----|-------------------------|----------------------|------|
| 1                    | ML   | M   | 111                     | 204                  | [38] |
| 2                    | HE   | M   | 152                     | 220                  | [38] |
| 3                    | PS   | M   | 134                     | 219                  | [38] |
| 4                    | FL   | M   | 126                     | 144                  | [38] |
| 5                    | RS   | M   | 116                     | 153                  | [38] |
| 6                    | IK   | M   | 99                      | 151                  | [38] |
| 7                    | EH   | M   | 108                     | 275                  | [38] |
| 8                    | KB   | F   | 103                     | 304                  | [38] |
| 9                    | HV   | M   | 84                      | 53                   | [38] |
| 10                   | Sci  | F   | 121                     | 104                  | [39] |
| 11                   | Def  | F   | 105                     | 57                   | [39] |
| 12                   | Tim  | M   | 144                     | 131                  | [39] |
| 13                   | HP   |     | 87                      | 18*                  | [40] |
| 14                   | AG   |     | 113                     | 169                  | [40] |
| 15                   | OL   |     | 133                     | 204                  | [40] |
| 16                   | RB   |     | 111                     | 234                  | [40] |
| 17                   | DA   |     | 136*                    | 325                  | [40] |
| 18                   |      | F   | 90                      | 15                   | [40] |
| 19                   |      | F   | 144† 102‡               | 116 725              | [41] |
| 20                   | Ch N | M   | 98                      | 200                  | [46] |
| 21                   | R N  | M   | 37                      | 141                  | [46] |
| 22                   | F N  | M   | 144                     | 141                  | [46] |
| 23                   | WH   | M   | 38                      | 153                  | [46] |
| Normal ( $\pm$ s.d.) |      |     | 130 $\pm$ 15            | 300 $\pm$ 50         | [44] |

Values corrected to 1.73 m<sup>2</sup> body surface

† Values in late pregnancy

‡ Values 6 months following delivery In this case studied early in pregnancy no glucose  $T_m$  was observed with plasma glucose concentrations of 888 mg/100 ml  $T$  glucose was 810 mg/min

decrease in the affinity of the substrate (glucose) for the transfer system could result in a shift of the curve to the right without altering  $T_m$ .<sup>1</sup> Thus the point at which the ratio  $T/T_m$  reaches 0.5 would occur at higher plasma concentrations of glucose. Changes in the permeability characteristics

<sup>1</sup> The amount of glucose reabsorbed per minute ( $T$ ) is calculated by subtracting the amount appearing in the urine per minute from the filtered load (plasma concentration  $\times$  glomerular filtration rate in milliliters per minute). It is apparent that  $T$  glucose represents a mean rate of tubular reabsorption since the amount reabsorbed decreases progressively along the proximal convoluted tubule. Until the maximal rate of reabsorption ( $T_m$ ) is reached, it is usually considered that  $T$  is a function of load. However, since the glomerular filtration rate does not significantly change in any one study as plasma glucose concentration is increased, then  $T$  may be considered a function of plasma glucose concentration. Although  $T$  does not represent true initial velocity for the reabsorption process, if the system follows Michaelis-Menten kinetics, then an approximate apparent  $K_m$  would be the concentration of plasma glucose when  $T/T_m = 0.5$  ( $1/V_{max} = 0.5$ ). This problem has been discussed by Walbrant [45].



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upon a reduction of renal threshold on the one hand or upon a decrease in the maximal capacity of the renal tubule to reabsorb glucose on the other

3 The majority of cases studied show a decrease in  $T_m$  glucose and a variable degree of 'splay' of the titration curve. It remains to be determined whether the abnormality is diffuse in all cases or the result of a heterogeneous population of nephrons in some cases

4 The fundamental mechanism of the renal tubular transport of glucose remains obscure, until more is known about this process the abnormality in renal glycosuria cannot be further defined

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detailed here. Many attempts have been made to define the nature of the underlying tubular defect and the resulting pathogenesis of the changes in blood and urine. From these studies has come general agreement on the clinical features and the over all pathogenesis of the disorder, but a more precise definition of the disease at the cellular or enzymatic level remains to be obtained. It is the purpose of this chapter to review current concepts of the nature of the tubular disorder in renal tubular acidosis and to discuss how this disorder may relate to the clinical manifestations of the disease.

## CLINICAL AND PATHOLOGIC MANIFESTATIONS

Since the major attention of this chapter is to be directed toward pathogenesis, the clinical syndrome of renal tubular acidosis will be only briefly summarized. Excellent descriptions of typical case histories are available elsewhere [8-10]. In general the syndrome has been described in two groups of patients—in infancy or in older children and adults. In infancy the clinical picture is quite non-specific and is often described under the all inclusive term "failure to thrive." There may be anorexia, nausea, vomiting, polyuria, dehydration, hypercalcemia, and fever. Nephrocalcinosis visible to x-ray examination is rare. If the patient is treated adequately, the ultimate prognosis is excellent. There is complete remission in the majority of cases [11]. This syndrome in infancy has not been so well studied as in the adult. It is not entirely clear that these two groups of patients have the same disorder.

In older children and adults, the constant acidosis (retention of  $H^+$ ) may be associated with non-specific complaints of anorexia, lethargy, fatigue, and failure of normal growth. The most serious complications relate to the loss of cations from the body, especially calcium and potassium. Depletion of calcium may be associated with rickets in childhood or osteomalacia in adults [9]. During the excessive urinary loss of calcium, local renal complications such as nephrocalcinosis and nephrolithiasis frequently occur. The excessive urinary loss of potassium may lead to recurrent attacks of weakness or even flaccid paralysis. The patient may have polyuria with impairment of urine concentration. The extracellular fluid pattern is that of a metabolic acidosis associated with hyperchloremia. In addition, studies may reveal hypokalemia or the chemical pattern in the serum of rickets or osteomalacia, i.e., low normal to low calcium, low phosphorus, and high alkaline phosphatase concentrations. In Fig. 39-1 is summarized the incidence of certain of the clinical features of renal tubular acidosis as found in 28 patients [7-10, 12-19]. The average age at the time of diagnosis in this series was 29, although symptoms often preceded diagnosis by a number of years. Patients are listed as having  $H^+$  depletion only if there were symptoms which

## Chapter 39

### Renal Tubular Acidosis

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*Lloyd H. Smith Jr*

Renal tubular acidosis is a descriptive term for a rare sometimes familial disorder of the renal tubule characterized by an inability to excrete a urine of normal acidity. This primary defect leads to a continuing hyperchloremic acidosis which is often associated with one or more secondary complications such as hypercalciuria with nephrolithiasis and nephrocalcinosis, rickets or osteomalacia, and severe potassium depletion.

Most of the disorders of renal tubular function which have been described as single entities are occasionally found as components of multiple tubular defects. This is true for renal tubular acidosis which may be one of the spectrum of abnormalities of the Fanconi syndrome [1] (cf Chap. 37). The syndrome of renal tubular acidosis may be closely simulated by the chronic administration of potent carbonic anhydrase inhibitors, a fact of considerable theoretical importance in speculations about its pathogenesis. Finally the extracellular fluid pattern of chronic hyperchloremic acidosis may be found following bilateral ureterosigmoid transplantations [2] or during the continued ingestion of large amounts of ammonium chloride. A similar pattern has been described in a group of patients with chronic pyelonephritis [3].

First descriptions of renal tubular acidosis were those of Lightwood in 1930 [4] and of Butler, Wilson and Earber in 1936 [5]. The syndrome was subsequently studied extensively by Albright and his collaborators with clarification of the major features of the overall physiologic defect in renal function [6]. From these studies plausible explanations for most of the systemic complications were offered and a rational therapeutic regimen was proposed. Over the last two decades the disorder of renal tubular acidosis has been recognized with increasing frequency and described under at least 14 separate terminologies [7] which will not be

tion reactions in intermediary metabolism. From a quantitative standpoint the lung with its feed back control adjustment for excreting this volatile anhydride of carbonic acid is the organ par excellence of acid base balance in that it can excrete a hundredfold more acid than the kidney and is capable of minute by minute adjustment of body pH. A second normal stress on the maintenance of pH is the metabolic liberation of certain acidifying anions of dietary origin particularly phosphate and sulfate. On an average dietary intake these represent approximately 50 to 100 mEq excess acid per day. It is in the controlled excretion of this fixed acid load that the kidney primarily serves to rid the body of excess hydrogen ions. The mechanisms by which the kidney serves this function can be arbitrarily divided into several categories. It will be apparent that these are in fact closely related phenomena: (1) excretion of sulfate and phosphate; (2) conservation of filtered bicarbonate; (3) tubular secretion of hydrogen ion; (4) tubular synthesis and excretion of ammonia.

#### *Excretion of Sulfate and Phosphate*

From many clearance studies it has been established that phosphate and sulfate are filtered by the glomerulus and partially reabsorbed by the tubule. Retention of the anions sufficient to produce acidosis is invariably associated with marked reduction of glomerular filtration. This is the type of renal acidosis found in most patients with uremia. If the retention of these anions were completely balanced by a corresponding retention of cations (largely the sodium ion) no acidosis would result. In the cross purpose of maintaining the constancy of intracellular and extracellular osmolarity this general increase of cation concentration is not allowable. These anions therefore displace the anions of weaker acids especially bicarbonate. Unless there is a corresponding reduction of carbonic acid mediated by the respiratory center and lung, acidosis is the inevitable result.

#### *Conservation of Filtered Bicarbonate*

The kidney is primarily concerned with the conservation of the corresponding buffer base (largely sodium) rather than the bicarbonate ion per se which is almost limitlessly available from metabolic carbon dioxide. It is evident that the maintenance of acid base balance is endangered by the profligacy of a glomerular filtration of approximately 45 moles bicarbonate per day, approximately fifteen times that normally present.

\* In the discussion to follow the terms acid and base are employed in the conventional clinical usage which is incorrect as chemical terminology [2, 3]. The terms imply the physiologic effects of certain anions and cations rather than their properties as proton donors or acceptors. Switching to more correct usage here would probably result in loss of clarity.

seemed clearly related to potassium loss. Other patients exhibited varying degrees of hypokalemia.

The few autopsy studies of the kidney in renal tubular acidosis have failed to reveal a constant pathologic picture. In general the glomeruli have been uninvolved. Interstitial calcification, evidence of pyelonephritis and vacuolization of tubular cells have been reported [6]. No abnormality comparable to the "swan neck" deformity of the tubule in the Fanconi syndrome has been described [20]. The tubular changes may be in part attributable to chronic potassium depletion [21].

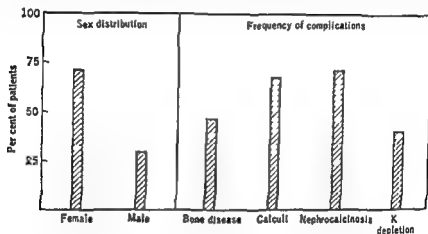


Fig. 39-1 The incidence of certain of the clinical features of renal tubular acidosis as found in 28 patients from the literature [10, 12, 15].

## PATHOGENESIS

Renal tubular acidosis is associated with a deficient acidification of the urine despite a continuing systemic acidosis. When the acidification mechanism is placed under additional stress by the use of acid or acid-forming salts, there is little or no further decrease in urine pH. Response of the kidney to systemic acidosis is therefore maximal but inadequate. Clearly the logical point of departure in investigating this syndrome has been the normal regulatory role of the kidney in acid-base balance in an attempt to determine in what manner this normal pattern is deranged.

### ROLE OF THE KIDNEY IN ACID-BASE BALANCE ✓

To attempt a general review of acid-base balance would be presumptuous in the present context. The following summary will relate only to the mechanisms by which the kidney contributes to the physiologic control of the hydrogen ion over the tenfold range compatible with life.

In man there is normally a constant tendency toward acidosis largely because of the liberation of carbon dioxide by certain specific decarboxyla-

tion reactions in intermediary metabolism. From a quantitative standpoint the lung with its feed back control adjustment for excreting this volatile anhydride of carbonic acid is the organ par excellence of acid base balance in that it can excrete a hundredfold more acid than the kidney and is capable of minute by minute adjustment of body pH. A second normal stress on the maintenance of pH is the metabolic liberation of certain acidifying anions of dietary origin particularly phosphate and sulfate. On an average dietary intake these represent approximately 50 to 100 mEq excess acid per day. It is in the controlled excretion of this fixed acid<sup>1</sup> load that the kidney primarily serves to rid the body of excess hydrogen ions. The mechanisms by which the kidney serves this function can be arbitrarily divided into several categories. It will be apparent that these are in fact closely related phenomena: (1) excretion of sulfate and phosphate (2) conservation of filtered bicarbonate (3) tubular secretion of hydrogen ions (4) tubular synthesis and excretion of ammonia.

#### *Excretion of Sulfate and Phosphate*

From many clearance studies it has been established that phosphate and sulfate are filtered by the glomerulus and partially reabsorbed by the tubule. Retention of these anions sufficient to produce acidosis is invariably associated with marked reduction of glomerular filtration. This is the type of renal acidosis found in most patients with uremia. If the retention of these anions were completely balanced by a corresponding retention of cations (largely the sodium ion) no acidosis would result. In the gross purpose of maintaining the constancy of intracellular and extracellular osmolarity this general increase of cation concentration is not allowable. These anions therefore displace the anions of weaker acids especially bicarbonate. Unless there is a corresponding reduction of carbonic acid mediated by the respiratory center and lung acidosis is the inevitable result.

#### *Conservation of Filtered Bicarbonate*

The kidney is primarily concerned with the conservation of the corresponding buffer base (largely sodium) rather than the bicarbonate ion per se which is almost limitlessly available from metabolic carbon dioxide. It is evident that the maintenance of acid base balance is endangered by the profligacy of a glomerular filtration of approximately 4.5 moles bicarbonate per day approximately fifteen times that normally present.

<sup>1</sup> In the discussion to follow the terms *acid* and *base* are employed in the conventional clinical usage which is incorrect as chemical terminology [22-23]. The terms imply the physiologic effects of cations and anions respectively rather than their properties as proton donors or acceptors. Switching to more correct usage here would probably result in loss of clarity.

in the entire extracellular fluid. This must be largely reabsorbed by tubular action. Evidence from several sources has indicated that glomerular filtrate is reabsorbed in the proximal segment of the tubule with little change in ionic concentration or pH [24]. It follows that five sixths of bicarbonate must be similarly reabsorbed here. It should be noted that some acidification has recently been demonstrated by micropuncture in the proximal tubule of the rat [25]. Acidification of the urine which occurs in the distal tubular segment, is associated with the reabsorption of most of the remaining bicarbonate in proportion to the final hydrogen ion concentration attained. It was previously postulated that the reabsorption of bicarbonate by these two segments occurred through different mechanisms—proximal reabsorption representing an active transport mechanism, distal reabsorption resulting from an ion-exchange reaction of  $H^+$  for  $Na^+$  with back diffusion of the released carbon dioxide [26]. Recently the use of more powerful carbonic anhydrase inhibitors has indicated that the reabsorption of bicarbonate is related to ion exchange at both sites [27], the essential difference lying in the ability of the distal tubular cell to concentrate  $H^+$  against a positive gradient. The reabsorption of bicarbonate is therefore intimately connected with the tubular secretion of  $H^+$ , which will be discussed below. Barring marked shifts in urine  $P_{CO_2}$ , the urine bicarbonate is a direct function of urine pH as signified by the Henderson Hasselbalch equation. "Bicarbonate wasting" therefore is roughly synonymous with defective acidification of the urine, as seen in renal tubular acidosis and in some patients with the more usual forms of chronic nephritis [28].

### *Tubular Secretion of Hydrogen Ions*

Hydrogen ion is concentrated in urine in excess of that found in plasma with the usual acid ash diet, and the concentration rises still further to compensate for systemic acidosis. This simple fact has long been known. The mechanism by which this is effected and controlled is still under investigation. Considerable information has been obtained by imaginative use of classical clearance techniques. Hydrogen ion secretion is part of the general phenomenon of ion exchange across cell membranes, an area of investigation which is just beginning to be approached in vitro.

For some time the assumption was made that acidification of the urine is effected by the selective tubular reabsorption of the more basic members of filtered buffer pairs—i.e.,  $Na_2HPO_4$  in preference to  $NaH_2PO_4$ . Hydrogen ions would therefore be progressively concentrated intraluminally. It was implicit in this theory that all  $H^+$  appearing in the urine has traversed the glomerular membrane. This conception of urine acidification was clearly demonstrated to be untenable in the classical studies of Pitts and Alexander [29]. Under their experimental conditions of acidosis and buffer infusions in dogs, it was shown that  $H^+$  appeared in



the urine (largely coupled with buffer as titratable acidity) in amounts considerably in excess of that which could be accounted for by glomerular filtration. The suggestion was made that the tubular cells secrete  $H^+$  into the lumen in exchange for  $Na^+$ . This exchange would allow a net loss of protons and simultaneously retrieve buffer base from the tubular urine. The source of  $H^+$  for this exchange was thought to be  $H_2CO_3$  produced by the hydration of carbon dioxide and catalyzed by carbonic anhydrase. The evidence in favor of such a pivotal function of carbonic anhydrase in urine acidification is indirect but impressive: a source of  $H^+$  within the renal tubular cells must be constantly available and capable of producing large amounts of exchangeable  $H^+$  (approximately 3 to 4 mEq  $H^+$  per min). Carbon dioxide as the major acid metabolically produced in the body would meet this requirement. Carbonic anhydrase is present in kidney cortex in high activity [30]. Finally a variety of chemicals known to inhibit carbonic anhydrase in vitro (notably sulfanilamide and more recently acetazolamide) interfere quickly and specifically with the renal acidification mechanism. No effects on other tubular functions have been detected [31].

The mechanism which controls the cationic exchange of  $H^+$  for  $Na^+$  across the cell membrane is not clear. The exchange in the proximal tubular segment is to be contrasted with that found in the distal segment as to both major function served and concentration gradient of  $H^+$  obtained. As previously pointed out the proximal exchange mechanism seems to be primarily designed to retrieve buffer base in the form of bicarbonate—perhaps four fifths or five sixths of that filtered being reabsorbed in this way. Micropuncture studies indicate that there is no change in pH in the proximal tubule, i.e. no net concentration of  $H^+$  [32]. Pitts has discussed the theory that the primary event is an active tubular reabsorption of  $Na^+$  in excess of  $Cl^-$  creating the observed negative intraluminal potential [33]. Hydrogen ions derived from carbonic acid would then simply diffuse into the lumen along an electrical gradient. Since there is no net concentration of  $H^+$  there is no associated diffusion of ammonia. This presumed diffusion of  $H^+$  seems to have no relationship to potassium secretion into the lumen. If in fact proximal reabsorption of bicarbonate is wholly dependent on  $H^+ Na^+$  exchange an impressive total of 20 to 30 mEq  $H^+$  per min must be produced and transferred by the proximal tubule.

The hydrogen ion-exchange mechanism in the distal tubular segment seems also to depend on the activity of carbonic anhydrase. In other respects it differs markedly from the simple diffusion thought to occur in the proximal segment. When maximally stimulated there is a concentration gradient of  $H^+$  of about eight hundredfold across the tubule (pH 4.6 in urine 7.4 in peritubular fluid). Although the exchange allows the salvage of the final one fifth or one sixth of bicarbonate buffer base

it also exchanges with the buffer bases of other filtered buffers, especially phosphate titrating them to a more acidic form. Finally the increased intraluminal  $H^+$  concentration appears to be a major factor leading to the diffusion of ammonia into the tubular urine. This will be discussed in more detail below. The nature of the active transport mechanism which can produce an eight hundred fold gradient of  $H^+$  across the inner cell membrane of the distal tubule has not been elucidated. Physiologic studies have firmly established, however, that the secretion of potassium into the distal tubular urine in exchange for sodium is competitively associated with the  $H^+$  transport mechanism; increased  $K^+$  secretion is produced by decreased  $H^+$  secretion and vice versa [27]. The limiting factor in  $H^+$  exchange in the distal tubule is that of a maximal concentration gradient (pH). When phosphate is infused during experimental acidosis and appears in the distal tubule to buffer the secreted  $H^+$  and thus to prevent the maximal pH gradient from being reached, the rate of  $H^+$  secretion increases markedly [29]. The maximal rate of  $H^+$  secretion by this mechanism has not yet been determined.

The fate of  $H^+$  secreted or diffusing into the tubular lumen depends wholly on the composition of the tubular urine at that point. The major products formed are  $H_2O$  from the decomposition of carbonic acid, the more acidic of buffer anionic pairs (especially  $H_2PO_4^-$ ), and the ammonium ion from the capture of  $H^+$  by diffused  $NH_3$ .

### *Tubular Synthesis and Secretion of Ammonia*

The central problem which the kidney faces in acid base balance is that of preserving buffer base endangered by glomerular filtration. Buffer base is represented by metabolic cations ( $Na^+$ ,  $K^+$ ,  $Mg^{++}$ ,  $Ca^{++}$ ) ingested as such and therefore of limited availability. The hydrogen ion is a cation which is metabolically produced and is therefore available for exchange reactions in the kidney to preserve buffer base as described above. Unfortunately  $H^+$  exchange is limited by the attending fall in pH. Ammonium is a second metabolically derived base which in effect serves to enhance the  $H^+$  exchange mechanism by neutralizing intraluminal  $H^+$ . The gradient is thereby maintained at less than the eight hundred fold maximum and  $H^+$  secretion can continue.

The source of urinary ammonia was clearly shown to be the amide group of glutamine and to a lesser extent the amino groups of other amino acids in classical studies by Van Slyke and his colleagues [34]. This has been widely confirmed. Data from many sources have indicated that three factors determine the amount of ammonia found in the urine: the availability of ammonia precursors, the cellular levels of enzymatic activities productive of  $NH_3$ , and the pH of the urine [35]. The influence of ammonia precursors is indicated by the increase of urinary ammonia found during the experimental infusion of glutamine or of a number of

other amino acids [36-37]. This presumably reflects a rise of intracellular ammonia concentration produced by increased substrate availability at a given enzyme concentration. Changes in substrate availability have not been shown to be of significance under other than experimental conditions. With continued acidosis there is a rise in urinary ammonia at a given pH. In the past this rise has been assumed to be due to an adaptive

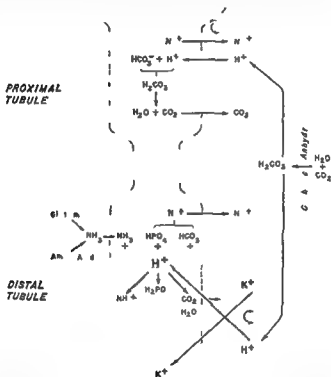


Fig. 33-2 Diagram to illustrate the function of the renal tubule in regulation of acid-base balance.

increase of enzymatic activity within the distal tubular cell. Such an adaptive increase of glutaminase in the presence of experimental acidosis has been directly demonstrated in the rat [38]. Recent studies in the dog, however, have failed to demonstrate any adaptive increase of the enzymes known to be associated with ammonia production [39]. Indirect evidence from the stimulatory effect of various ammonia precursors during chronic ammonium chloride ingestion in man suggests enzymatic adaptation [37]. The mechanism by which this isohydric increase of ammonia production is brought about in man remains uncertain.

Urinary ammonia is generally found to be inversely proportional to

urine pH, at any particular stage of adaptation to acidosis. Evidence has been presented that ammonia, formed within the cell, passes into the tubule by free diffusion down a concentration gradient [35]. This is but one example of the nonionic diffusion of weak bases and acids across the renal tubule [40]. In alkaline urine equilibrium is attained so that the amount of ammonia passing into the urine is limited. In an acid urine ammonium ion is formed. The removal of free ammonia permits diffusion to continue. Seemingly the intraluminal reaction of  $H^+$  and  $NH_3$  serves to further the excretion of each by maintaining the necessary concentration gradient for ion exchange and free diffusion respectively.

This review of the role of the kidney in acid base balance has of necessity been abbreviated and overly didactic. Figure 39.2 summarizes diagrammatically many points in the above discussion. In summary the kidney must rid the body of the nonvolatile anionic residues of metabolism with the minimal expenditure of buffer base. This is carried out by the substitution within the tubular lumen of metabolically derived cations ( $H^+$  and  $NH_4^+$ ) for the buffer base which has been placed in jeopardy by glomerular filtration.

#### NATURE OF THE PRIMARY DEFECT IN RENAL TUBULAR ACIDOSIS

Renal tubular acidosis is the result of failure of the normal renal mechanisms for the defense of acid base balance. Glomerular filtration remains unimpaired unless secondary complications of nephrocalcinosis, nephrolithiasis and pyelonephritis lead to renal parenchymal damage. The

'fixed acids' (sulfate and phosphate principally) do not accumulate in extracellular fluid. They are normally excreted—but at an increased cost in buffer base. Despite a continuous systemic acidosis and in the face of an administered acid load there is bicarbonate wasting and inability to secrete  $H^+$  and  $NH_3$  into the tubular urine. In brief the glomerular component of renal acid base defense is normal; the tubular component is defective.

In the previous section evidence was presented that bicarbonate reabsorption proximal or distal is intimately related to the hydrogen ion-exchange mechanism for sodium. In general in renal tubular acidosis there is a normal direct relationship of urine bicarbonate and urine pH, there being no consistent alteration of urine  $P_{CO_2}$ . These facts being so, bicarbonate wasting seems not to be a primary defect but merely a reflection of an inadequacy of the tubular secretion of  $H^+$ .

The most consistent abnormality found in renal tubular acidosis is an inability to excrete urine of sufficient acidity in the face of systemic acidosis. Stated more precisely, the renal tubule does not exchange sufficient  $H^+$  to retrieve the necessary amount of buffer base. This is reflected in a decreased urinary titratable acidity. Normally minimal urine pH is about 4.5; in renal tubular acidosis it is usually found to be

60 to 68 From previous considerations of the  $H^+$  secretion mechanism several hypotheses concerning the defective acidification could be advanced (1) the tubule is unable to produce and secrete  $H^+$  sufficiently rapidly for normal function of the exchange mechanism (2) the tubule is unable to attain the usual maximal trans-tubular gradient of  $H^+$  of 800:1 (3) there is a failure of the proximal  $H^+$ -exchange mechanism which would result in diminished proximal bicarbonate reabsorption and consequent flooding of a normally functioning distal  $H^+$  secreting mechanism The three hypotheses will be considered here briefly

Studies of the response of the kidney to intravenous phosphate infusions have been carried out by several groups of investigators This procedure which increases the available urinary buffer (i.e. acceptor anion for  $H^+$ ) has regularly resulted in a marked increase of  $H^+$  secretion by the tubule in renal tubular acidosis usually with some decrease in urine pH [41 42 13] Rates of  $H^+$  secretion are produced which if sustained would easily correct the systemic acidosis These studies suggest that the kidney in renal tubular acidosis is quite capable of forming and secreting  $H^+$  at a rate commensurate with the amount of buffer base to be reabsorbed if this exchange can occur at a reduced  $H^+$  concentration gradient On the other hand the defective tubule cannot continue to secrete  $H^+$  in the face of a trans-tubular gradient of greater than approximately 80:1 rather than the normal maximal gradient of 800:1 In oversimplified terminology the defect in renal tubular acidosis seems to be primarily one of concentration rather than production or transport of  $H^+$

The hypothesis was advanced by Latner and Burnard [41] on the basis of studies of renal tubular acidosis in children that the defect lies in the proximal reabsorption of bicarbonate and that this obscures by neutralization a normally functioning distal acidifying mechanism This hypothesis was based largely on the following two observations (1) the infusion of neutral phosphate buffer led to a marked increase of  $H^+$  secretion with a considerable fall in urine pH (2) urine  $P_{CO_2}$ 's were found to be elevated in renal tubular acidosis and were considered to reflect the excessive formation of carbonic acid from  $HCO_3^-$  and  $H^+$  in the distal tubule It was suggested that in some way phosphate increased the proximal reabsorption of bicarbonate and thereby unmasked a more normally functioning distal  $H^+$  secretion mechanism The hypothesis of Latner and Burnard can be challenged The infusion of phosphate buffer at pH 7.4 regularly results in a fall of urine pH in normal subjects The explanation of this phenomenon has remained obscure [43] Urine  $P_{CO_2}$ 's have not been uniformly elevated in determinations on adult patients Finally if the proximal reabsorption of bicarbonate were the only tubular defect the infusion of bicarbonate in such patients should further tax the incompetent reabsorptive mechanism and result in an exacerbation of bicarbonate wasting This experiment has been carried out It was found that

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temic hyperchloremic acidosis. Because of this fact it has been speculated that renal tubular acidosis represents a tubular deficiency of carbonic anhydrase perhaps on a genetic basis. There is no direct evidence to support this hypothesis; its chief attraction is that of analogy. Two experimental findings moreover provide evidence against it.

Carbonic anhydrase is a metalloprotein enzyme the only known function of which is to accelerate the attainment of equilibrium of the reaction



It does not affect the equilibrium concentration of carbonic acid (and hence of its dissociation product  $\text{H}^+$ ) but only the rate at which this equilibrium concentration is reached. Previously cited evidence from phosphate infusions demonstrates that the rate of secretion of  $\text{H}^+$  by the tubule in renal tubular acidosis is adequate given an acceptor anion to allow this secretion to continue without an adverse shift in the  $\text{H}^+$  gradient. This implies that the rate of  $\text{H}^+$  production within the cell (presumably under the influence of carbonic anhydrase) is not a limiting factor in the acidification of the urine. It is difficult to visualize how carbonic anhydrase could influence the ability of the tubule to secrete  $\text{H}^+$  against a concentration gradient. Secondly, studies in several patients with renal tubular acidosis have shown a response to a carbonic anhydrase inhibitor acetazolamide with a further rise in urine pH and bicarbonate excretion [44]. Whether this is a normal response is still under investigation. It suggests the presence of carbonic anhydrase and its function in producing whatever urinary acidification is attained in these patients. This one finding would not exclude a quantitative deficiency of the enzyme. Taken in conjunction with the evidence that the rate at which  $\text{H}^+$  can be secreted under proper conditions is not limiting it suggests that renal tubular acidosis cannot be attributed to a primary deficiency of carbonic anhydrase. It is unlikely that the primary defect in renal tubular acidosis can be more rigorously defined until additional information is available concerning the biochemical and biophysical mechanisms involved in normal  $\text{H}^+$  exchange across the cell membrane.

#### **PATHOGENESIS OF THE COMPLICATIONS OF RENAL TUBULAR ACIDOSIS**

A brief summary of some of the clinical findings in renal tubular acidosis has already been given. It remains to review whether plausible explanations for these complications of the disease can be based on a presumed single primary tubular defect in  $\text{H}^+$  secretion.

##### *Nephrocalcinosis and Nephrolithiasis*

It has been well shown that patients with renal tubular acidosis exhibit hypercalciuria with a tendency toward hypocalcemia [6]. The administra-

infused bicarbonate was reabsorbed normally in renal tubular acidosis [42-43]. A localized proximal tubular defect would therefore appear to be untenable with the data at hand.

There is still disagreement as to whether there is a primary defect in ammonia excretion in renal tubular acidosis. In the many measurements, urinary ammonia has almost invariably been found reduced considerably below that ordinarily found in comparable systemic acidosis without kidney disease. For the urine pH attained, however, the urinary ammonia in

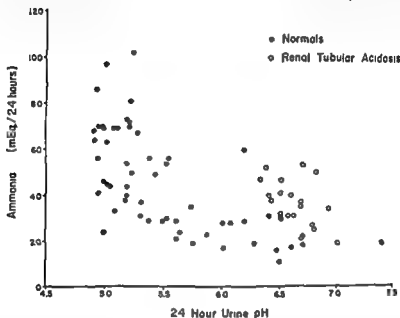


Fig. 39-3 Comparison of 24 hr urine pH and ammonium content in patients with renal tubular acidosis and in control subjects [15]. (By permission of the American Journal of Medicine.)

renal tubular acidosis is normal or perhaps even increased as compared to controls with acute acidosis (Fig. 39-3). In comparison with chronic experimental acidosis ammonia excretion may be somewhat diminished [37]. The close relationship between  $H^+$  concentration and the diffusion of nonionic  $NH_3$  into the urine has been previously emphasized. No determinations of glutaminase or other enzymes involved in ammonia production have been carried out on the kidney in renal tubular acidosis. All in all, the primary defect appears to lie in some aspect of  $H^+$  secretion and concentration, ammonia production being secondarily reduced because of failure to establish more favorable conditions for nonionic diffusion.

Carbonic anhydrase is the only enzyme which has been implicated in the tubular formation and secretion of  $H^+$ . Inhibition of carbonic anhydrase causes a reduction in tubular  $H^+$  secretion and may result in  $pH$ -



temic hyperchloremic acidosis. Because of this fact it has been speculated that renal tubular acidosis represents a tubular deficiency of carbonic anhydrase perhaps on a genetic basis. There is no direct evidence to support this hypothesis; its chief attraction is that of analogy. Two experimental findings moreover provide evidence against it.

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##### *Nephrocalcinosis and Nephrolithiasis*

It has been well shown that patients with renal tubular acidosis exhibit hypercalciuria with a tendency toward hypocalcemia [6]. The administra-

tion of ammonium chloride increases the hypercalcaemia, alkali therapy quickly reduces it. The mechanism by which the renal tubular defect results in an increased clearance of calcium is not apparent largely because knowledge concerning the renal excretion of calcium is incomplete. It has been suggested that calcium is lost as one of the 'buffer bases' because of inadequacy of urine acidification [9]. On the other hand the tubular reabsorption of calcium has not been demonstrated to be dependent upon an exchange reaction with  $H^+$ . Hypercalcaemia occurs over long periods of time in a urine which is neutral or minimally acidic, creating favorable conditions for the precipitation of the calcium phosphate stones so frequently found. It has been reported that urinary excretion of citrate is low in renal tubular acidosis, another factor which would tend to favor stone formation [40]. The cause of nephrocalcinosis is not so apparent. Here calcification occurs in the renal parenchyma in a disorder associated with a decreased tendency toward precipitation of bone salts in osteoid (see below) and seemingly an increased clearance (hence a decreased tubular reabsorption) of calcium. It has been reported that acetazolamide reduces urine citrate excretion and may produce renal calcinosis in the rat [46].

### *Rickets or Osteomalacia*

Depending upon the age of the patient, rickets or osteomalacia may be found. A major and continuing source of calcium loss in the urine has been outlined above. The resulting tendency toward hypocalcaemia is thought to serve as a stimulus to increased secretion of parathyroid hormone, which acts on the renal tubule to decrease the reabsorption of phosphate and directly on bone to increase its rate of destruction. Dissolution of bone salts is also fostered by continued acidosis per se. Bone is an important source of available buffer base. Finally, there is evidence to suggest that the action of vitamin D is partially inhibited during systemic acidosis [47]. The result of these interrelated events is decreased bone because of both increased destruction and delayed calcification of osteoid. As a consequence bone pain or deformity may be the most striking disability associated with renal tubular acidosis.

### *Hypokalaemia*

Balance studies on patients with renal tubular acidosis have exhibited an increased urinary loss of potassium. In keeping with current concepts of potassium excretion by the kidney this probably represents tubular secretion of  $K^+$  in exchange for sodium. The competitive nature of the  $H^+$  and  $K^+$  exchange function in the distal tubule has been previously noted. A diminished ability to secrete  $H^+$  should therefore lead to an increased secretion of  $K^+$  by the tubule. On the other hand, repair of the acidosis by administering alkali further inhibits  $H^+$  secretion but reduces

rather than increases urinary  $\text{K}^+$  [9] In renal tubular acidosis other factors may be at play The tendency toward a negative  $\text{Na}^+$  balance may lead to a secondary stimulation of aldosterone secretion which in turn would act to increase the urinary loss of potassium [48] Potassium deficiency has been well documented with hypokalemia typical electrocardiographic changes and attacks of weakness or of flaccid paralysis—all responding quickly to replacement therapy A number of patients with renal tubular acidosis have exhibited hyposthenuria and decreased responsiveness to antidiuretic hormone In the past this has been attributed to renal damage by calcification or pyelonephritis More recently the suggestion has been offered that this may relate to the clear cell nephropathy of potassium deficiency with the resulting well-documented loss of renal concentrating ability [21]

The above summary indicates that the major clinical manifestations of renal tubular acidosis relate directly or indirectly to excessive renal loss of cations This is consistent with a single renal tubular defect characterized by an inability to concentrate hydrogen ions in the urine

### FAMILIAL INCIDENCE

The inclusion of renal tubular acidosis in a compilation of inborn errors of metabolism may be legitimately criticized Of all the clinical patterns of renal tubular dysfunction it has shown least evidence of being a hereditary disorder [49] The case histories of more than 50 patients with renal tubular acidosis have been published but the familial occurrence of the syndrome has been limited to the following few reports Schreiner Smith and Kyle [7] described renal tubular acidosis with nephrocalcinosis and nephrolithiasis in two brothers The 9 year old son of one of these patients was found to have hyperchloremia rickets and nephrocalcinosis The patient's father gave a history of repeated attacks of renal colic and exhibited mild hyperchloremic acidosis Unfortunately he refused to cooperate in further studies The disorder occurred in two siblings whose case histories have been published by Foss Perry and Wood [8] The first patient was a girl of 17 with dwarfism rickets nephrocalcinosis nephrolithiasis and a history of attacks of muscle weakness suggestive of hypokalemia Her brother who was seen at age 14 was unusually short with retardation of epiphyseal development and a history suggestive of repeated attacks of renal colic Typical chemical changes of renal tubular acidosis were found in blood and urine The father of these two patients was found to have renal calculi a urine pH of 6.8 and a rather low plasma bicarbonate level The occurrence of the syndrome in twins was reported briefly by Rendle-Short [50] One of the infants died before it was established whether or not the twins were identical The other twin was said to have made a complete recovery

In a report by Cooke and Kleeman of several patients with renal tubular acidosis a footnote was added to state that nephrocalcinosis had been found in a sibling of one of their patients [51]. No further details of this family have been reported. Engel attributed to sulfonamide intoxication renal tubular acidosis in two siblings who subsequently made complete recoveries [52]. Wilansky and Schneiderman have described a 33 year old woman with renal tubular acidosis whose father died at 60 of kidney disease with nephrocalcinosis and whose brother killed accidentally at age 20 was found to have shrunken, calcified kidneys [12]. Most patients with the syndrome do not have a history to suggest a familial occurrence. Similarly most studies of the immediate relatives of such patients have failed to detect a familial pattern. In general these surveys have been limited to a search for overt complications of the disorder such as nephrocalcinosis, nephrolithiasis, or bone disease, there has been no testing for a possible heterozygous defect such as a reduced ability to respond to an exogenous acid load. The fragmentary information available does not allow resolution of the mode of inheritance of those few cases of renal tubular acidosis which have clearly been familial. The presence of the disorder in two and possibly three successive generations suggests a dominant trait.

The rarity of a familial pattern has led to other speculations concerning etiology. It has been suggested that chronic pyelonephritis may produce in the occasional patient a selective damage to the renal acidification mechanism [6]. It is true that urinary tract infection is frequently found in this group of patients who often exhibit nephrolithiasis or nephrocalcinosis or both. Many patients especially those recognized in childhood have not had demonstrable infections. These children often seem to make a complete recovery from the disorder contrary to the adult patients. It seems more plausible to consider infection as a complication of the renal injury rather than a factor of primary etiologic importance. The simulated renal tubular acidosis produced by carbonic anhydrase inhibitors has led to the theory that sulfonamides may irreversibly damage the renal tubule to impair permanently the normal acidification mechanism. Most patients do not have a history of sulfonamide ingestion. The widespread use of more powerful carbonic anhydrase inhibitors (such as acetazolamide) over a period of a number of years has led to a few isolated reports of complicating nephrolithiasis [53] but no permanent interference with tubular function.

In summary the etiology of renal tubular acidosis in the majority of cases remains obscure. A familial occurrence has been well documented in a few incidences. Other theories of etiology such as selective renal damage from pyelonephritis or carbonic anhydrase inhibitors, have failed to account for the appearance of the disease. It is possible that a more careful search of the families of patients especially with provocative

tests such as the renal response to ammonium chloride would demonstrate a more consistent pattern of inheritance

## SUMMARY

1 Renal tubular acidosis is one of the more frequently encountered of the rare disorders of renal tubular function. It may occur as a single defect or as one component of multiple tubular defects as in the Fanconi syndrome.

2 Physiologic studies suggest that the pathogenesis lies in an inability of the tubule to attain an intraluminal hydrogen ion concentration sufficient to effect normal conservation of buffer base. Whether there is an additional defect in ammonia production has not yet been established.

3 The complications of this disorder relate to excessive urinary loss of cations particularly calcium and potassium. Calcium loss leads to rickets or osteomalacia and local complications in the kidney such as nephrolithiasis and nephrocalcinosis. Potassium loss is associated with muscle weakness and potassium deficiency nephropathy. When a compensatory amount of buffer base is supplied in the diet these complications are effectively prevented.

4 In a small number of patients renal tubular acidosis has been found as a familial disorder. Current information does not allow resolution of the mode of inheritance but the possible occurrence in three successive generations suggests a dominant trait.

5 Although studies over the past two decades have indicated the general outline of the renal defect and have led to a rational therapy the precise nature of the derangement in hydrogen ion secretion has yet to be elucidated.

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## Chapter 40

### Vasopressin resistant Diabetes Insipidus

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*Jack Orloff and Maurice B. Burg*

Vasopressin resistant diabetes insipidus is a rare congenital and familial disease characterized by failure of the kidneys to respond to antidiuretic hormone. The essential features of the disorder are polyuria, polydipsia, and the excretion of persistently hypotonic urine. The disease was first described by Forssman [1] and Waring et al. [2] who, recognizing the inefficacy of vasopressin in promoting the excretion of hypertonic urine, distinguished it from diabetes insipidus due to insufficiency of neurohypophyseal hormone. Later, Williams and Henry [3] introduced the name *nephrogenic diabetes insipidus* in order to emphasize that insensitivity of the renal tubule cells to antidiuretic hormone is responsible for all the clinical findings in the disease.

#### PHYSIOLOGY OF WATER BALANCE

In normal man the osmotic pressure of plasma is essentially a reflection of the ratio of water to solute in the body, is virtually constant (285 to 290 mOsm per kg  $H_2O$ ) despite wide variations in the intake of solute and water. Regulation is achieved by varying both the volume flow of urine and its osmotic pressure in response to changes in plasma osmolality. A fall in plasma osmolality, indicative of relative water excess, is normally attended by the excretion of large volumes of urine less concentrated than plasma. This loss of water without its equivalent content of solute (285  $\mu$ Osm for every milliliter of water) restores plasma osmolality to normal. Conversely, hyperosmolality, whether due to water loss or retention of solute in excess of water, is corrected by the excretion of urine more concentrated than plasma.

The precise adjustment of urine flow and osmolality is in large part due to the remarkable efficiency of the pituitary-renal system. The kidney is capable of varying urine flow under physiologic conditions in the adult



from less than 1 to more than 20 ml per min and urine concentration from a maximum of 1400 to a minimum of 30 to 40 mOsm per kg  $H_2O$ . These changes are effected to a considerable degree by variations in the tubular reabsorption of water largely determined by the action of the neurohypophyseal hormone vasopressin.

#### ROLE OF THE NEUROHYPOPHYSIS IN WATER BALANCE

Vasopressin, an octapeptide [4] is elaborated in the supraoptic and paraventricular nuclei of the hypothalamus [5].<sup>1</sup>

Vasopressin is thought to be stored in the posterior portion of the pituitary body from which it is secreted into the blood in response to nervous stimuli originating in cells (osmoreceptors) in the anterior hypothalamus and transmitted to the pituitary along the supraopticohypophyseal tracts [6]. On the basis of Verney's [6] classical demonstrations it has been concluded that shrinkage of the osmoreceptors due to an elevation in the effective osmotic pressure<sup>2</sup> of plasma effects the release of antidiuretic hormone (vasopressin) into the circulating blood. The resultant increase in urine osmolality restores plasma osmolality to normal thereby diminishing the rate of secretion of hormone. Conversely, a reduction in effective osmotic pressure by virtue of swelling of the osmoreceptors leads to inhibition of antidiuretic hormone release and permits the excretion of large volumes of dilute urine. It is important to recognize that graded release of hormone is essential in order to afford the continuous regulation of urine flow and concentration necessary to minimize deviations in plasma osmolality. Although the renal regulation of water balance is inadequate in the absence of a properly functioning hypothalamohypophyseal system or when the kidney is insensitive to the effect of antidiuretic hormone as in the disease under discussion the patient with either form of diabetes insipidus is capable of maintaining plasma osmolality within the normal range merely by adjusting fluid intake as long as the volume of water ingested exceeds that excreted in the urine by an amount equivalent to that dissipated in the form of

<sup>1</sup> The hormone isolated from ox pituitary by du Vigneaud and his associates is a cyclic octapeptide with the following structure [ ]



Hog vasopressin contains lysine in the penultimate position. All other mammals studied including man are thought to secrete the arginine form depicted above.

<sup>2</sup> Effective osmotic pressure is that exerted by solutes that do not penetrate cell membranes rapidly. Sodium and attendant anions represent the major osmotically effective constituents of the extracellular space. Urea on the other hand though it may contribute appreciably to the total osmotic pressure does not affect the distribution of water between cells and surrounding extracellular fluids since it penetrates cells freely and its concentration is approximately equal in the two phases.

insensible loss and perspiration excessive concentration of body fluids will not occur. In this situation, thirst rather than variable reabsorption of water assumes a regulatory role.

### RENAL REGULATION OF WATER BALANCE

Although the antidiuretic effect of vasopressin and the physiologic factors involved in its release from the hypophysis have been recognized for many years, the intrarenal events which transpire in the elaboration of hypotonic or hypertonic urine are not yet completely understood.

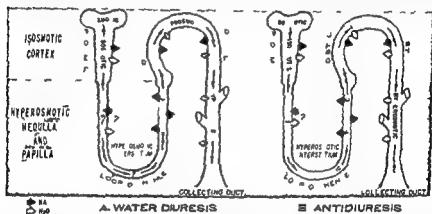


Fig. 40-1 Schematic representation of the nephron during water diuresis (A) and antidiuresis (B). The diagram is described in detail under The Countercurrent Theory. Although not indicated in the figure, the longitudinal interstitial osmotic gradient increases progressively from corticomedullary junction to papilla. The size of the arrows representing  $H_2O$  and  $NaCl$  reabsorption indicates the relative extent of these processes. The development of hyperosmolality in the descending limb of Henle's loop has been established only in antidiuresis. That this also occurs in water diuresis is likely but unknown. Furthermore, although hyperosmolality in the descending limb is probably the result of water abstraction, the movement of urea and other solutes from the interstitial space into the lumen also may be an important factor. The role of urea in the concentrating and diluting processes discussed in the text has been omitted from the figure.

Sufficient data derived from a variety of sources are available to justify a tentative description of the process [7-13].

In order to account for the normal variations in urine flow and concentration, the plasma ultrafiltrate formed at the glomerulus must be modified as it courses down the tubule. Two fundamental processes are involved: the selective abstraction of solute and the tubular reabsorption of water. Water reabsorption in the tubule is divided into at least three and possibly four functionally and anatomically distinct phases. On the basis of the most recent evidence, it is believed that water diffuses<sup>1</sup>

<sup>1</sup> The term diffusion is used throughout to connote movement of water across the tubular membrane. It should be recognized that this is inaccurate since bulk flow through pores along an osmotic gradient may account for most of the net movement [18].

passively out of the proximal segment the descending limb of Henle's loop the distal convoluted tubule and the collecting duct along osmotic gradients established by prior solute removal. The proximal segment and descending limb of Henle's loop are considered to be freely permeable to water under all circumstances the distal convolution and collecting duct are freely permeable only in the presence of antidiuretic hormone. Consequently variations in the permeability of the membrane to water in the latter segments impose restrictions on diffusion and permit the elaboration of urine of varying tonicity and volume. Furthermore it has been established [14] that there is a progressive increase in the osmotic pressure of the renal interstitial fluid from cortex to papilla (Fig 40-1), the medulla and papilla being hyperosmotic to plasma under all circumstances whereas the cortex is isosmotic.

Normally 20 per cent of the plasma perfusing the kidney is filtered at the glomerulus. Of the ultrafiltrate 80 to 85 per cent is reabsorbed in the proximal segment essentially as an isosmotic solution [7 8 12 15]. As noted above the luminal membrane in this area is freely permeable to water under all circumstances. Sodium and attendant anion are reabsorbed by a process involving active sodium transport (i.e. transport against an electrochemical gradient) water then diffuses passively out of the tubule along the resultant osmotic gradient. The reduced volume of isosmotic fluid is then delivered to the loop of Henle. Although the sequence of events in this segment has not been established with certainty it is probable that Wirz's [11 14] original contention that the urine is progressively concentrated as it flows downward in the descending limb and is then diluted in the upward ascent in the ascending limb is correct. In support of this are the observations (1) that urine at the tip of Henle's loop is hypertonic to plasma [12] \* and (2) that urine in the first portion of the distal convolution and therefore presumably in the ascending limb of Henle's loop is hypotonic to plasma irrespective of the final urine concentration [12 15]. In view of this it is reasonable to assume that the descending limb is freely permeable to water under all circumstances hypertonicity being effected by loss of water without solute into the surrounding hypertonic interstitium whereas the ascending limb is impermeable to water dilution being achieved by the active extrusion of sodium chloride without water. It is essential to recognize that the processes described thus far are uninfluenced by vasopressin and occur as described whether or not the final urine is concentrated or dilute. Were no other factors involved one could visualize the situation in vasopressin resistant diabetes insipidus (and in water diuresis in general) as the excretion of most of the residual hypotonic urine leaving

This has been established in antidiuretics only. No examinations of loop fluid during water diuresis have been reported. In the context of this discussion it is assumed that hypertonicity in this segment is also developed in water diuresis.

the loop of Henle. That this is an oversimplification is attested to by arguments to be presented subsequently.

The sequence of events in the distal convolution and collecting duct, unlike that in the proximal portions of the nephron, differs considerably in water diuresis and antidiuresis. In the absence of vasopressin and when a dilute urine is to be excreted, the luminal membrane in the distal nephron is thought to be relatively impermeable to water. Dilute urine which leaves Henle's loop is rendered more hypotonic by the continued abstraction of solute without water in the distal convolution and perhaps even in the collecting duct [16, 17]. Consequently urine less concentrated than plasma is excreted into the bladder. Although some loss of water along its osmotic gradient takes place in the distal nephron even in the absence of vasopressin, it is generally insufficient to effect the excretion of either an isotonic or hypertonic urine.

In marked contrast, when vasopressin is present hypertonic urine is elaborated. Antidiuretic hormone is thought to increase the permeability of the distal convolution [9-11] and collecting duct to water [11], so that for all intents and purposes these areas of the tubule function as do the proximal segment. Although electrolyte is abstracted from hypotonic urine in the distal convolution just as in water diuresis, water now diffuses freely out of the tubule until osmotic equilibrium is achieved. The remaining urine, once again isotonic to plasma, is delivered to the collecting duct where water in excess of solute is lost by movement into the hypertonic interstitium [10]. The resultant concentrated urine is excreted.

An effect of vasopressin on membrane permeability has been deduced from studies in other tissues. Koefoed-Johnsen and Ussing [18] observed a marked increase in net water movement along an osmotic gradient across isolated frog skin under the influence of neurohypophyseal extract. They concluded that the hormone produces this effect by increasing the size of pores within the skin through which water may flow. It is likely that vasopressin induces similar changes in membrane permeability in both the distal convolution and collecting duct. Wirz [10] and Gottschalk and Mylle [12] noted that urine in the distal convolution which remains hypotonic throughout during water diuresis (when antidiuretic hormone secretion is in abeyance) becomes isotonic in this segment in antidiuresis. An analogous increase in the permeability of the collecting duct to water has also been suggested by Wirz [11]. In the presence of antidiuretic hormone the osmotic pressure of collecting duct contents approximates that of the surrounding tissue and blood. Consequently, as noted above, urine made dilute by solute abstraction in Henle's loop no longer flows through essentially water impermeable conduits in the distal nephron. Instead, water in excess of solute moves out of the two areas ultimately resulting in the elaboration of hypertonic urine.

The view that water movement in all segments of the nephron including that in the collecting duct is passive has only recently been proposed. The terminal process which results in the elaboration of urine more concentrated than plasma had been assumed to be an active process since it apparently required uphill transport of water i.e. movement of water from a solution in which the activity of water is less than that of the surrounding interstitium. The argument was based on the belief that the osmotic pressure of the renal interstitial space approximated that of all other tissues of the body and was 290 mOsm per kg H<sub>2</sub>O. Wirz [14] resolved this problem and provided a rational basis for considering that water movement out of the tubule lumen is passive (along its activity gradient) even when urine is markedly hypertonic. He observed by direct cryoscopic analysis that the solute concentration of kidney water increases progressively from cortex to papilla and that both the inner medulla and papilla are markedly hypertonic to peripheral plasma. He concluded that the hypertonic environment surrounding the collecting duct provides the osmotic force necessary for passive movement of water out of this segment. He was the first to suggest that the achievement of diffusion equilibrium when the membrane is made freely permeable to water by vasopressin results in the elaboration of hypertonic urine. It is a testimony to Wirz's ingenuity to recall that hypertonicity of the medulla had been noted earlier [16] only to be ignored by most renal physiologists.

### *The Countercurrent Theory*

Wirz [15] considered the hypertonicity of the medulla and papilla (the longitudinal concentration gradient) to be due to deposition of sodium chloride transported out of the ascending limb of Henle's loop into the surrounding interstitium. Although not explicitly stated as such in his original description it is likely as noted above that loss of water without solute in the descending limb raises the concentration of the urine in this segment above that of plasma. This process concomitant concentration and dilution of urine in opposing segments of a loop like structure in which the flow of urine in one segment is counter to that of the other has a particular physiologic advantage. Since the NaCl concentration of ascending limb urine is from the beginning approximately equal to that of the environment the transport of sodium salts out of this segment is nowhere against a steep concentration gradient. This minimizes any energetic difficulties which might arise were sodium salts transported from hypotonic urine to a markedly hypertonic environment as has been suggested by Berliner et al [13].

Both the anatomic arrangement of the loop and the processes described have all the features of a hairpin countercurrent multiplier system. The principle of the multiplier has been described in detail by Hargitay and Kuhn [19] and was originally applied in a somewhat modified fashion

to the kidney by the workers and Wirz [14]. It is evident that deposition of NaCl in excess of water in the interstitial space of the medulla and papilla will raise the osmotic pressure of this area above that of the cortex. Progressive multiplication of this longitudinal gradient to the extent observed experimentally is visualized in the following manner. In a hypothetical first circulation through the loop, the intraluminal fluid has the same concentration throughout equal to that of the cortex. Active transport of NaCl from the water impermeable ascending limb lowers the concentration of ascending limb contents and raises that of the interstitial fluid and descending limb (see above). The slightly hypertonic descending limb fluid moves around through the hairpin bend in the loop into the ascending limb temporarily abolishing the concentration gradient between the opposing segments. However, continuing transport of NaCl from the ascending limb now containing fluid more concentrated than in the previous circulation, raises the osmotic pressure of the interstitial and descending limb fluids once again. Descending limb fluid more concentrated than previously moves into the ascending limb, and the process continues until in the steady state the longitudinal gradient is multiplied manyfold, whereas the small transverse gradient between loop contents and interstitium is at each point in the renal medulla equal and unchanged. In other terms the hairpin countercurrent multiplier system, by permitting transport against a minimal gradient provides the basis for multiplying the small gradient between loop contents and interstitium manyfold in the longitudinal axis.

Whether or not urinary dilution in the ascending section of the loop and concentration of the interstitial spaces occurs as described and is accomplished by a hairpin countercurrent multiplier device, sequestration or trapping of NaCl in the interstitial space in order to maintain the concentration gradient cannot be explained simply on the basis of this hypothesis. Since blood courses through this area one would expect the excess solute to be dissipated and the solute concentration never appreciably to exceed that of the inflowing blood. This aspect discussed by Wirz [20] has been emphasized and developed in detail by Berliner et al. [13]. Noting that the postglomerular capillaries in this area do not course directly through the kidney but also bend back upon each other in the shape of loops they suggested that the capillaries may act as countercurrent exchangers. This is illustrated in Fig. 40-2. Free diffusion of solute from the outflowing to the inflowing limb of the capillary loop permits recirculation of solute in the medulla minimizing the loss of excess NaCl and other solutes (notably urea) from the area. The concentration gradient is thereby maintained and not dissipated as it would be were blood flowing through in one direction only. The capillary countercurrent exchanger not only maintains the interstitial NaCl concentration indirectly providing the osmotic force necessary for the terminal con-

concentrating process but also serves to trap any urea which diffuses into the interstitial space from the collecting duct [21]. The concentration of urea in medullary water approaches that of collecting duct contents being approximately equal in antidiuresis. In view of this Berliner et al [13] have pointed out that in so far as collecting duct urea is osmotically balanced by urea in the surrounding interstitial fluid it will increase the

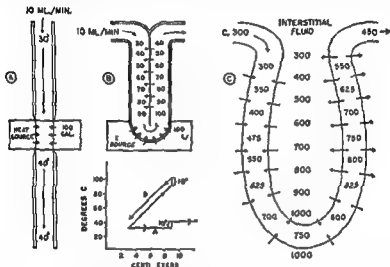


Fig 40-2 Principle of the countercurrent exchanger (Reproduced from Berliner et al [13] with permission of the author and publisher.)

A and B represent the effect of heating water flowing at the rate of 10 ml per min through a pipe. The addition of 100 cal per min raises the temperature 10° in both A (straight flow) and B (countercurrent flow). However, since the incoming water is heated by the outgoing water in B, the maximum temperature attained in the countercurrent system is considerably higher than with straight flow. The graph compares the temperature along the flow tubes in each system.

C, countercurrent flow as applied to the capillary loop, showing that it is not necessary for the limbs of the loop to be in direct contact. In the hypothetical illustration given, both limbs are in contact with the same interstitial fluid of progressively increasing concentration. Sodium salts (arrows) at first enter the capillary blood, later partly return to the interstitial fluid. Note the analogy between B, in which heat is recirculated, and C, in which sodium salts are similarly retained in an area.

osmotic pressure of urine beyond that attained by simple equilibration of collecting duct contents with interstitial NaCl. In other terms, the urine urea does not need to be balanced by an equivalent concentration of NaCl in the interstitial fluid, as do the other solutes. Consequently, final urine osmolality will be greater or lesser depending on the urea load presented to the collecting duct, even though no change in interstitial NaCl content occurs. The clinical significance of this is considerable. Inadequacy of urea load may limit the apparent concentrating ability of

the kidney, though no defect in either the permeability of the membrane to water or in the so-called sodium "pump" may be present [21-22].

A schematic drawing of the nephron is illustrated in Fig. 40-1. The cortex containing the glomerulus and the proximal and distal convolutions is represented as being isosmotic with peripheral plasma. The medulla and papilla in which the loops of Henle, the collecting duct, and the deeper portions of the capillary loops are embedded are represented as being hyperosmotic to plasma. Vasopressin affects only the distal convolution and the collecting duct. In diabetes insipidus or whenever vasopressin is either ineffective or not secreted, urine made dilute by transport of electrolyte out of both Henle's loop and the distal convolution loses only a small fraction of its water as it passes through the distal portions of the nephron and is excreted as such in the bladder. In the presence of vasopressin, free permeability of the distal convolution and collecting duct membrane to water permits osmotic flow of water out of the lumen. Consequently the osmolality of urine in each segment approximates that of the tubular environment, becoming isosmotic to plasma in the distal convolution and hyperosmotic in the collecting duct. It is important to recognize that the major effect of vasopressin in so far as water balance is concerned is in the distal convolution. The promotion of osmotic equilibrium in this area and the formation of isosmotic urine results in the restoration of as much as  $\pm 15$  per cent of the filtered water to the body, whereas the reduction in volume in the collecting duct, though resulting in urine hypertonicity, restores considerably less ( $\pm 5$  per cent).

#### FACTORS OTHER THAN VASOPRESSIN

##### *Effects of Filtration Rate*

In order properly to evaluate the significance of changes in urine flow and osmolality in patients suspected of having vasopressin-resistant diabetes insipidus, it is essential to recognize the influence of at least two other factors: filtration rate and solute excretion on the concentrating process. Berliner and Davidson [23] have demonstrated that a reduction in filtration rate may, even in the absence of vasopressin (or at least when its secretion is minimal and constant), promote the excretion of hypertonic urine. Presumably volume flow of hypotonic urine to the collecting duct is significantly diminished by this procedure. Consequently, although outward movement of water in the terminal segment is minimal under these circumstances, it may be sufficient to concentrate the small volume of urine delivered to the collecting duct. In view of this observation, minor increases in urine osmolality in diabetes insipidus following dehydration need not necessarily signify residual antidiuretic hormone activity. On the other hand, it is not at all certain that the elaboration of hypertonic



urine in patients with the pituitary disorder following protracted dehydration or manipulations designed to reduce filtration rate can uniformly be ascribed to the fall in filtration rate itself. Residual antidiuretic hormone activity can never be completely excluded except in patients with the severe form of vasopressin resistant diabetes insipidus. In one such patient although urine osmolality rose during dehydration in association with a fall in filtration rate it never exceeded that of plasma [24].

### *Effects of Solute Excretion*

The influence of solute excretion on urine flow and concentration is clinically of greater significance than the effect of filtration rate. Osmotic diuretics diminish water and solute reabsorption in the nephron and result in the excretion of large volumes of essentially isosmotic urine whether or not vasopressin is acting. Both sodium transport and water reabsorption are interfered with in the proximal segment because of the osmotic restraint of nonabsorbed intraluminal solute. Consequently a larger than normal volume of isosmotic urine enters the more distal portions of the nephron overwhelming the capacity of the segment to modify the concentration of the urine. Urine osmolality rises in osmotic diuresis both in patients with nephrogenic diabetes insipidus [25] and in normal persons [26] during antidiuretic hormone suppression whereas in hydropenic subjects in whom vasopressin is acting the converse a progressive fall in osmolality occurs [27]. In both instances urine osmolality approaches that of unmodified glomerular filtrate as the fraction of proximal urine delivered to the distal segment increases.

It is important to emphasize that no fundamental processes other than proximal reabsorption need be disturbed during osmotic diuresis to account for the observations. It is probable that in the presence of vasopressin more water is actually removed in the collecting duct during solute diuresis than at low rates of solute excretion [28]. That this does not result in the achievement of maximal hypertonicity is due in part at least to the resultant dilution of the medullary interstitial space. Even were osmotic equilibrium to obtain in this segment which is unlikely during the rapid flow of solute diuresis the maximal osmotic pressure which could be obtained would be considerably less than normal.

The effects of osmotic diuresis are more easily visualized if one considers as do Wesson and Anslow [10] that hypotonic urine is made up of two hypothetical moieties: (1) an isosmotic portion (referred to as the osmolar clearance) equal in volume to the number of milliliters of fluid containing all the urine solute at the concentration of plasma; and (2) an additional amount of distilled water (referred to as solute-free water) equal in volume to that which would have to be added to the isosmotic moiety to reduce its concentration to that observed. Hypertonic urine on the other hand is

depicted as being made up of an isosmotic portion less that amount of solute-free water which would have to be abstracted from it to produce the observed hypertonicity (referred to as negative solute-free water). In this view, the removal of solute from isosmotic filtrate (the process of urinary dilution) results in the elaboration of solute free water, and conversely the abstraction of solute-free water from isosmotic filtrate results in the addition of negative-free water to the interstitium and the production of hypertonic urine. Clearly, even were the amount of solute-free water formed in water diuresis or the amount of negative free water abstracted in antidiuresis unchanged in osmotic diuresis, urine concentration would approach isotonicity asymptotically with increasing urine flow since an increasingly greater fraction of the final urine would be composed of what may be considered nonabsorbed proximal isosmotic fluid.

In the light of these concepts it is not possible to assess the clinical significance of changes in urine concentration without consideration of the rate of solute excretion. In this regard the *posthenuria* of renal insufficiency as has been pointed out by Baldwin et al [29] and Platt [30], may be a consequence of the high rate of solute excretion relative to the diminished filtration rate and does not necessarily denote either an inability to concentrate the urine or a vasopressin unresponsiveness. The filtered load of urea per nephron is markedly increased in azotemia limiting the capacity of the kidney to elaborate either a maximally hypertonic or hypotonic urine. The extremes of urine osmolality are observed only when solute excretion per nephron is minimal.

Even in the absence of antidiuretic hormone as noted above, water may diffuse out of the relatively impermeable distal segment and collecting duct although at a considerably lesser rate than when vasopressin is present [20-31]. In the course of solute diuresis in vasopressin resistant diabetes insipidus Orloff and Walker [20] noted (Fig 40-3) not only a progressive rise in urine osmolality but also an increasingly greater rate of solute free water excretion. They concluded that this phenomenon is in part due to a progressive decline in the rate of outward diffusion of water initially freed in Henle's loop and the distal convolution as a consequence of the osmotic restraint of intraluminal solute. Thus the presence of solute in tubule urine decreases the activity of water and thereby its rate of outward diffusion in both the distal convoluted tubule and collecting duct. Even in the presence of vasopressin maximal solute diuresis may so limit outward diffusion of water as to result in the excretion of hypotonic urine. Results of this nature have been observed during vasopressin administration in patients with pituitary diabetes insipidus [32] and in dogs undergoing combined water and solute diuresis [31]. This is not a reflection of insensitivity to vasopressin but indicates that despite relatively free permeability to water in the distal nephron the reduction in both the transit time and the osmotic gradient prevents the attainment of diffusion equilibrium and permits the delivery of large volumes of

hypotonic rather than isotonic urine to the terminal concentrating site. In view of these observations it is important to determine the normal relationship between urine osmolality and solute excretion before ascribing isosthenuria (see above) or even hyposthenuria<sup>5</sup> to true vasopressin insensitivity.

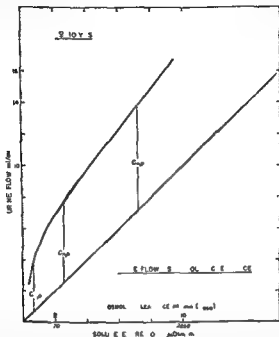


Fig 40-3 The relationship between urine flow and solute excretion in a 10-year-old girl with vasopressin resistant diabetes insipidus. The diagonal line represents the excretion of isotonic urine; the curvilinear line that observed experimentally. The difference between the hypothetical and observed lines represents a solute-free water excretion ( $C_{H_2O}$ ) which rose progressively throughout the study.

## CLINICAL ASPECTS

Vasopressin resistant diabetes insipidus manifests itself soon after birth, but since polyuria in infancy is rarely considered a symptom of disease even in the first born of the most apprehensive parent, it is frequently undiagnosed in early life. Furthermore the presenting signs which in fact may be alarming are often nonspecific, being those associated with severe dehydration: fever, convulsions in infants, vomiting, constipation, and hyperosmolality. The characteristic syndrome—polydipsia, polyuria, and persistent hyposthenuria (specific gravity generally

The excretion of urine less concentrated than plasma

less than 1 005<sup>6</sup>)—diagnostic in offspring of affected families is noted only when hydration is adequate Under these circumstances, other causes of dehydration can generally be excluded

### RESPONSE TO VASOPRESSIN

In contrast to what occurs in diabetes insipidus of pituitary origin, neither the copious urine flow nor the low urine concentration is affected by the administration of large doses of vasopressin (Table 40-1) Nor is it

TABLE 40-1 LACK OF EFFECT OF PURIFIED VASOPRESSIN IN A 12 YEAR-OLD MALE WITH VASOPRESSIN RESISTANT DIABETES INSIPIDUS

| Time min | V ml/min   | U m | P m | GFR ml/min |
|----------|--|-----|-----|------------|
| 0        | Infuse 10 mg/min insulin in 6 l ml/min 5% dextrose |     |     |            |
| 50-70    | 10.2   | 44  | 233 | 83         |
| 70-90    | 10.8   | 48  | 293 | 87         |
| 130      | Infuse 50 mU/min vasopressin                       |     |     |            |
| 130-150  | 11.1   | 44  | 290 | 87         |
| 150-170  | 10.7   | 48  | 287 | 89         |

Note V = urine flow U = and P = urine and plasma osmolality mOsm/kg H<sub>2</sub>O GFR = glomerular filtration rate

possible to effect a diminution in flow or the elaboration of a hypertonic urine by stimuli known to enhance secretion of vasopressin from the pituitary body This is illustrated in Table 40-2 Although severe dehydration may ultimately result in a rise in urine osmolality and an associated fall in urine flow urine hypertonicity has not been observed in patients totally unresponsive to vasopressin under these circumstances [24-33]

Mild forms of the disease, characterized by partial responsiveness to vasopressin may exist in females of affected families Carter and Simp

\* Specific gravity measurements have been used for many years to estimate urine concentration Since the former is a measure of urine density rather than of solute concentration and is dependent on both the weight and volume displacement of urine solids it cannot be used as a precise estimate of osmotic pressure This can be determined only by measuring the concentration of osmotically active constituents in urine by determining either the freezing point or vapor pressure of the urine Despite this there is a reasonably good correlation between urine specific gravity and osmotic pressure Significant discrepancies are observed only when abnormal solutes (glucose protein) constitute a large fraction of the total urine solute Under these circumstances specific gravity gives a falsely high estimate of urine concentration The converse a low estimate has been reported by Miles et al [34] when urea is present in high concentration in urine The limits of urine osmolality as determined by cryoscopic analysis have been referred to earlier Patients with nephrogenic diabetes insipidus generally cannot elaborate urine more concentrated than 50 to 100 mOsm although during solute diuresis urine osmolality may approach isotonicity (280 mOsm) as a limit Urine specific gravity is less than 1 005 approaching 1 010 during osmotic diuresis

kiss [35] observed that female siblings and relatives of patients with the full blown disorder were able to concentrate their urine to approximately 1 018 to 1 019 following 12 hr of dehydration. Although this is significantly less than normal it does represent a considerable degree of responsiveness. *Forsman [1] has reported similar findings. Furthermore Childs and Sidbury [36] have stated that two mothers of patients with the disease responded minimally to exogenous vasopressin. Neither was able to elaborate a hypertonic urine. Apparently although full expression of the renal defect may occur in both females and males incomplete excretory capacity has thus far been reported only in females.*

TABLE 40-2 ABSENCE OF EFFECT OF DEHYDRATION IN 12 YEAR-OLD MALE WITH VASOPRESSIN RESISTANT DIABETES INSIPIDUS

| Time min | I ml/min   | U m | P m | GFR ml/min |
|----------|--|-----|-----|------------|
| 0        | Weight = 32.9 kg   |     |     |            |
| 60       | Infuse 20 ml/min insulin in 100 ml/min 5% dextrose in H <sub>2</sub> O |     |     |            |
| 100-164  | 6.2  | 84  | 285 | 69         |
| 164-186  | 5.7  | 81  | 285 | 71         |
| 186-27   | 7.3  | 78  | 233 | ■          |
| 227-67   | 7.7  | 79  | 293 | 79         |
| 267-308  | 7.8  | 79  | 236 | 80         |
| 308      | Weight = 30.9 kg   |     |     |            |

Note: I = urine flow; U m and P m = urine and plasma osmolality mOsm/kg H<sub>2</sub>O; GFR = glomerular filtration rate.

In all forms of the disease dehydration and its consequences are the most frequent complications as well as the most common causes of death. The high infant mortality in affected families is undoubtedly ascribable to this. The reported association of hydronephrosis and bladder hypertrophy in some affected individuals is probably secondary to polyuria and voluntary retention.

#### MENTAL AND PHYSICAL RETARDATION

A number of authors have commented on the relatively high incidence of mental and physical retardation in children with this disorder [3, 37-42]. It is not at all clear that these are primary accompaniments of the renal defect. More likely structural brain damage if present is secondary to repeated episodes of severe dehydration [38, 39] and to the altered growth pattern occasioned by inadequate food intake because of the exhausting influence of uncontrolled polydipsia. *Hallman et al [42] observed that an infant suffering from the disease became so exhausted from polydipsia when subsisting on a regular diet that he was unable to eat and play normally. Mental performance and nutritional status were*

improved by altering the protein and salt content of the diet so as to decrease obligatory solute and water loss. The reduction in polyuria diminished the time required for drinking and enabled the child to play, eat, and sleep adequately.

### RENAL FUNCTION

The only physiologic abnormality present in the disease is defective water reabsorption. In all other respects renal function may be considered to be normal. Renal blood flow, glomerular filtration rate, glucose, phosphate and amino acid transport, as well as other discrete tubular functions, may be unaffected [2, 3, 25, 37, 41]. Changes in filtration rate and plasma flow which have been observed by some authors may have been due either to dehydration or associated but unrelated structural disease. Abnormalities of acid base balance and electrolyte metabolism are excluded by the normal plasma electrolyte patterns observed when hydration is adequate.

### PATHOGENESIS

The pathogenesis of the disease is unknown. Antidiuretic substance has been found in the blood [41, 43] and urine [40, 44, 45] of patients, indicating that endogenous hormone is produced. Furthermore the administration of large doses of vasopressin, which is without effect on urine flow and concentration, results in abdominal pain and blanching, known side effects of the hormone [41, 44, 46, 47]. This indicates that rapid inactivation of the hormone within the body is not responsible for the disease. No information is available relative to the normal *in vivo* metabolism of vasopressin in man. It has not been ascertained whether the hormone may be inactivated in an abnormal fashion by the pertinent tubule cells. In view of recent evidence that vasopressin is bound to renal tissue during antidiuresis [45], it is possible that normal "binding" does not occur in vasopressin-resistant diabetes insipidus. Other preparations of vasopressin, the synthetic arginine and lysine forms, are also ineffective [47].

No structural basis for the disorder has been found. Gross and microscopic examination of renal tissue by the usual techniques has not been revealing [2, 37, 40, 41, 49]. Although MacDonald [41] reported some shortening of the proximal segment in a microdissected specimen from one child, Darmady [49], who has examined one infant and two adults with the disease, has not been able to find any abnormality by microdissection or by histologic techniques.

On the basis of all the studies, it is assumed that end-organ unresponsiveness (presumably in the distal convolution and collecting duct) is responsible for the inefficacy of the hormone. The resultant fixed im-

permeability of the distal nephron to water probably does not differ from that normally occurring in man during water diuresis since the relationship between solute excretion and urine osmolality is the same in both groups [47]

## DIAGNOSIS

The diagnosis is established without difficulty in the adequately hydrated subject in whom no response to vasopressin is observed. Other causes of isosthenuria or hyposthenuria may be mistaken for the incompletely expressed form of the disease. However the characteristic findings of the underlying disorder should preclude errors in diagnosis. Diminished ability to concentrate urine maximally has been observed in hypokalemic nephropathy [50], hypercalcemia [51], sickle cell disease [52], so-called water losing nephritis [53], and postobstructive uropathy [54]. In all but the last of these although the urine is hypertonic to plasma following vasopressin it is not maximally concentrated. Isosthenuria in renal insufficiency is due in large part to solute diuresis (see above) and is generally not attended by true unresponsiveness to vasopressin. Although it is possible that some patients may have structural disease with resultant insensitivity to vasopressin this has not been established. Whether the reports of decreased concentrating ability in tubular acidosis [55] and in congenital tubular defects associated with aminoaciduria [56] are indicative of true unresponsiveness to vasopressin cannot be established on the basis of the available evidence.

Psychogenic polydipsia may be associated with diminished responsiveness to vasopressin [57]. DeWardener [58] observed a reversible limitation in concentrating ability in these and in normal subjects following prolonged chronic and excessive water ingestion. DeWardener [57] has also stated that patients with the pituitary disorder may demonstrate this on occasion. The cause of the temporary alteration in tubule permeability is unknown. Prolonged administration of vasopressin and water in both man and dog may also be associated with an apparent diminution in responsiveness to the hormone [59-61].

### *Hereditary Pituitary Diabetes Insipidus*

Although the distinction between pituitary diabetes insipidus and the renal form generally is not difficult, mild cases of vasopressin resistant diabetes insipidus have been confused with hereditary pituitary diabetes insipidus [1]. The latter disorder is familial and also appears in early infancy. However it is a less severe disease than the fully expressed form of vasopressin resistant diabetes insipidus. Consequently dehydration and retarded development are uncommon. Nonspecific atrophy of the posterior lobe of the pituitary and hypoplasia of the paraventricular and

improved by altering the protein and salt content of the diet so as to decrease obligatory solute and water loss. The reduction in polyuria diminished the time required for drinking, and enabled the child to play, eat, and sleep adequately.

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supraoptic nuclei have been observed at autopsy. According to Forssman [1] two forms of this disease exist, one transmitted as a sex linked recessive the other as an autosomal dominant.

## GENETICS

The renal form of diabetes insipidus is an inherited disease which appears with greater frequency in male members of affected families than in females. It was originally considered by Forssman [1] and Williams and Henry [3] to be transmitted as a sex linked recessive characteristic. All of the 17 patients examined by these authors were males whose parents and female siblings were thought to be unaffected. Furthermore the female siblings repeatedly passed the trait on to their male offspring. No example of male to male transmission was observed. It has since been established that the disease is not completely recessive in female heterozygotes [3a]. Although all the males thus far reported exhibit complete unresponsiveness to vasopressin, mild forms of the disease have been observed in female siblings and relatives of affected males [1, 3a]. In addition in families in which there is no evidence that the fathers were affected several females have been reported in whom the disease is fully as severe as in males [3a, 44, 62, 63]. On the basis of these observations it appears that nephrogenic diabetes insipidus is a dominant sex linked disorder with variable expressivity in heterozygous females. If this is so mothers and half of the sisters of affected males should either be carriers of the defect or exhibit some form of the disease. More careful studies in the future may establish this. In the only affected families studied with this in mind 12 of 17 females examined were unable to concentrate their urine normally following dehydration [3a]. The discrepant preponderance of male patients in other sibships may indicate either a low degree of penetrance in females, a failure to recognize cases with low expressivity, or lowered viability and fertility of those affected males whose daughters would otherwise provide the excess female morbidity. The severity of the symptoms in all the males reported in the literature may be indicative of a high degree of expressivity in this sex. However no systematic study has excluded the possibility that male hemizygotes may show variable expressivity between and within kindreds as do heterozygous females.

Childs and Sidbury [36] although conceding that the disease is most reasonably viewed as due to a gene occupying the X chromosome and showing perhaps intermediate dominance have suggested an alternative hypothesis: an autosomal dominance with some degree of sex limitation. The latter view of the mode of inheritance has been emphasized by Cannon [62]. He described a large pedigree in which 4 of 55 affected males were reported to have affected sons. Cannon explained this pedigree by assuming that in some kindreds at least the disease is an autosomal

dominant with low penetrance in females. Allen [64] has pointed out that Cannon's pedigree conforms closely to sex-linked inheritance with the exception of the 4 cases of male to male transmission. If inheritance were autosomal, half the male offspring of affected males should have the disease and only half the female offspring of such males should be carriers. According to Cannon's pedigree chart, only 6 of the 44 male children were affected and of 19 females who had children 16 were affected or had affected children. Either of these discrepancies alone is sufficient to preclude an autosomal theory of inheritance in this family.

## TREATMENT

There is no specific therapy for the disease. Adequate hydration easily achieved in the adult by allowing free access to water is essential to prevent the deteriorating effect of repeated episodes of dehydration. Obligatory water loss may be minimized by reducing solute intake [39-42]. This, however, is rarely necessary except in infants. It has been reported recently that chlorothiazide, a potent diuretic, may induce satisfactory remissions in this disorder [65].

The therapy of the acute episode of dehydration may differ from that in other situations. If sufficient water can be administered orally, hyperosmolality of body fluids is easily overcome. On the other hand, if parenteral therapy is necessary, 5 per cent dextrose in water, although the fluid of choice in other instances of dehydration, may aggravate the hypertonicity. Orloff and Walser [47] observed that the rapid infusion of isosmotic dextrose in water in nephrogenic diabetes insipidus, in so far as it produces solute diuresis (glycosuria), will so increase solute-free water excretion as to promote the development of hyperosmolality and cellular dehydration. The dextrose is not metabolized with sufficient speed to release solute-free water in excess of that excreted in the urine. It is apparently virtually impossible to maintain positive water balance if 5 per cent dextrose is administered to patients with this disorder. Successful correction of hyperosmolality may be accomplished by administering 2½ to 3 per cent dextrose in water, since under these circumstances it is possible to limit the extent of the urinary solute and water loss. It is advisable to administer water without solute orally as soon as is clinically feasible.

## SUMMARY

1. Vasopressin-resistant diabetes insipidus is a renal disorder characterized by polydipsia, polyuria, and the excretion of persistently hypotonic urine. It is distinguished from diabetes insipidus due to in-

Intravascular hemolysis is not observed if 2½ to 3 per cent dextrose in water is administered parenterally. Less concentrated solutions may be hazardous.

sufficiency of antidiuretic hormone by a lack of response to exogenous vasopressin

2 All males thus far reported are completely unresponsive to antidiuretic hormone. Mild forms of the disease, in which a limited response to vasopressin occurs, are observed in some female members of affected families

■ The only known defect present in the disease is insensitivity of the renal tubule cells to vasopressin. No anatomic or biochemical basis for the end organ unresponsiveness has been determined. The hormone normally accelerates the passive reabsorption of water in the distal nephron ultimately resulting in a reduction in urine flow and a rise in urine osmolality. In its absence, or when the pertinent tubule cells are insensitive to its effect, water diuresis is continuous

4 The only established treatment is maintenance of water balance by ingestion of adequate amounts of water. In infants it is sometimes necessary to reduce the water requirement by restricting solute intake. Life expectancy is normal if episodes of dehydration are prevented by permitting free access to water under all circumstances. Chlorothiazide has induced remissions in some cases

5 The genetic pattern of the disorder is consistent with transmission as a sex-linked dominant characteristic with variable expressivity in females. The suggestion that an autosomal form of inheritance may be present in some families is not fully supported by the available data

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## Chapter 41

### Glycinuria

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*James B Wyngaarden*

In 1957 DeVries Kochwa Lazebnik Frank and Djaldetti [1] reported a family in which excessive urinary excretion of glycine was found in association with nephrolithiasis. The glycinuria appeared to be due to a selective renal tubular defect of glycine reabsorption and was not accompanied by unusual loss of other amino acid phosphate or glucose. A kidney stone obtained from one patient was composed chiefly of calcium oxalate but also contained a small amount of extractable glycine. To date this is the only report of what is probably a very rare disease.

#### CLINICAL SYNDROME IN THE INDEX CASE

This disorder was first recognized in a 20 year old woman whose initial urologic symptoms occurred at 2 years of age when she had fever associated with pus in the urine. The first of a series of attacks of renal colic occurred 3 years later. Renal lithiasis was recognized roentgenographically at age 6. One pyohydronephrotic kidney was removed at 11 and 8 years later a calculus developed in the remaining kidney. Examination then showed many calcium oxalate crystals in the urine but no infection. When the patient was 19 pyelotomy was performed and the stone was removed. On a high fluid intake the urine subsequently showed a trace of albumin but no cells casts or crystals.

#### METABOLIC STUDIES IN GLYCINURIC SUBJECTS

The urine of the patient was found to contain unusual amounts of glycine but normal quantities of other amino acids. Initial studies were performed by two-dimensional paper chromatography. Quantitative estimations of the amount of glycine in the urine were obtained by a microbiologic assay. Six members of the patient's family were also

examined and glycine excretion was abnormally high in three. Two family members also gave a history of renal colic. Data on these family members are shown in Table 41-1. It will be noted that abnormal glycine excretion was found in a total of four individuals, all women representing three successive generations.

TABLE 41-1 DATA ON GLYCINURIC FAMILY

| Family members              | Age<br>yr | History        |  | Glycine<br>in urine<br>mg/24 hr | Glycine<br>in serum† |
|-----------------------------|-----------|----------------|--|---------------------------------|----------------------|
|                             |           | Renal<br>colic | Stones<br>passed or<br>diagnosed<br>(including<br>x ray) |                                 |                      |
| Propositus                  | 40        | +              | +  | 111                             | Normal               |
| Mother                      | 48        | -              | -  | 516                             | Normal               |
| Father                      | 52        | -              | -  | 145                             |                      |
| Sister                      | 22        | +              | +  | 63                              | Normal               |
| Grandmother (maternal side) | 75        | +              | -  | 593                             |                      |
| Uncle (maternal side)       | 55        | -              | -  | Normal†                         |                      |
| Daughter of sister          | 2         | -              | -  | 10                              |                      |

Microbiologic assay

† Two-dimensional chromatography

SOURCE DeVries et al [1]

TABLE 41-2 INTRAVENOUS GLYCINE LOADING STUDY

| Subject    | Urinary free glycine load, mg |         |
|------------|-------------------------------|---------|
|            | 0 min                         | 200 min |
| Normal     | 40                            | 334     |
| Normal     | 50                            | 340     |
| Glycinuric | 800                           | 1240    |

Each subject received 1 gm glycine per 10 lb body weight in 70 ml saline solution within 2½ min.

SOURCE DeVries et al [1]

The serum level of glycine in three glycinuric individuals was normal as judged by a semiquantitative paper chromatographic evaluation. Consequently it was believed that the glycinuria could not be attributed to overflow secondary to hyperglycinemia. A disturbance of renal tubular reabsorption of glycine was therefore postulated. Studies of glycine excretion following intravenous infusion of glycine showed a significantly greater urinary load of glycine in the original patient than in two normal subjects similarly investigated (Table 41-2). The urinary

glycine excretion of the glycinuric subject was not influenced by 11 days of ingestion of a diet containing only 30 gm protein per day

The stone which was obtained was composed mainly of calcium oxalate but free glycine was extractable from the stone at room temperature with 1 N HCl and proved to be of the order of 0.3 per cent of the dry weight of the stone. No glycine was found in eight random oxalate stones obtained from other patients. The possibility of an abnormal degree of conversion of glycine to oxalate via glyoxylate (cf Chap 14) was considered but the urine contained only 18 to 40 mg oxalate per day on nine consecutive days and glyoxylic acid could not be demonstrated in her urine.

Additional studies on two members of this family revealed normal calcium excretions on a regular diet, normal responses to intravenous calcium infusions and normal phosphorus clearances. No glycosuria or abnormal xanthinuria was detected in the proband. Except for the maternal grandmother, no family members showed evidence of decreased bone density by roentgenographic study. Thus these subjects appeared to exhibit a disorder different from those reported by Evered [2] whose patients showed glycinuria, hypophosphatemia, hyperphosphaturia and prolinuria associated with osteomalacia (cf Chaps 36 and 37).

## SOME ASPECTS OF NORMAL GLYCINE METABOLISM

Glycine is abundantly present in the diet and is found in all proteins. It is a nonessential amino acid and is readily synthesized by the majority of living organisms. The principal source of nondietary glycine in the animal body is serine [3, 4] which can be readily transformed into glycine by loss of its  $\beta$  carbon as formyl. A second source is by transamination of glyoxylic acid from glutamic acid. Glycine can also be generated by catabolism of sarcosine or of ingested threonine.

Glycine is an important compound in many biosynthetic pathways. In addition to its role in protein synthesis and its ready transformation into intermediates of carbohydrate metabolism, glycine is directly involved as a precursor of purines, porphyrins, glyoxylic and oxalic acids, serine, glutathione, sarcosine, creatine and glycocholic acid. It is found in urine as the benzoyl peptide, hippuric acid, and also forms conjugates with certain steroids, valicylates, and Bromsulfalein.

Estimates of the glycine pool have been made in rats and more recently in man. Arnstein and Neuberger [5] estimated that the readily available glycine pool of the rat is of the order of 100 mg per kg body weight. Watts and Crawhall [6] estimated the glycine pool in man to be 80 mg per kg on the basis of measurements of the specific activity of free urinary glycine following administration of glycine  $C^{14}$ . Gutman, Yu, Black, Yalow, and Berson [7] estimated the glycine pool in man to be

83 mg per kg from the ratio of isotope incorporation of glycine 1 C<sup>14</sup> into urinary uric acid under tracer and load conditions

The glycine pool is not homogeneous. Arnstein and Neuberger [5] have evidence for a second glycine pool in rats which is in slow equilibrium with the first or readily available glycine pool. Glycine was withdrawn from it in response to large doses of benzoate. Benedict, Kalinsky, Scarrone, Wertheim and Stetten [8] observed that there are significant differences in labeling of hippurate and the sarcosine moiety of creatine in dystrophic subjects following oral administration of glycine N<sup>15</sup>. They suggest that there are at least two glycine pools in poor equilibrium with one another. Also, Watts and Crawhall [6] observed a greater isotopic labeling of the glycine moiety of hippurate than of free urinary glycine in man following oral administration of glycine 1 C<sup>13</sup>. Presumably, the hepatic glycine pool was preferentially labeled under this circumstance.

The rate of turnover of glycine has been estimated in several species. In the rat and guinea pig the daily biosynthesis of glycine was estimated to be about 2 and 3 gm per kg per day respectively [3, 6] and these values were not significantly influenced by restriction of glycine intake [3] or by vitamin B<sup>12</sup> or ascorbic acid deficiency, but were reduced in folic acid deficiency [9]. Watts and Crawhall [6] estimated the turnover of glycine in man to be only 1.0 gm per kg per day.

The plasma levels of glycine normally range from 1.15 to 1.92 mg per 100 ml as determined by resin column chromatography [2, 10] and 1.47 to 2.83 mg per 100 ml as determined chemically [11]. By the latter method the values for whole blood are higher (1.76 to 3.02 mg per 100 ml) because of the greater concentration of glycine in the red cell (1.64 to 3.73 mg per 100 ml). Variations of concentration of glycine in plasma, blood or red cells have been reported in disease [11]. Plasma glycine levels decrease significantly (20 to 32 per cent) following oral ingestion of 10 gm sodium benzoate [12].

## RENAL HANDLING OF GLYCINE

### *Normal Mechanisms*

The normal urinary excretion of free glycine is 70 to 200 mg per day as determined by column chromatographic [2, 10] or microbiologic methods [1]. A considerably larger amount of glycine is excreted in conjugated form and is released by acid hydrolysis [2, 10]. The total glycine excretion may normally amount to 800 to 1,125 mg per day.

Glycine clearance has been estimated in only a few subjects. It ranges from 1.5 to 5.7 ml per min [2]. Presumably glycine is freely filtered at the glomerulus and quite efficiently reabsorbed in the renal tubules. The mechanism by which it is reabsorbed is not well understood. Such studies as are available have been performed chiefly in the dog. Pitts's [13] data indicate that glycine and creatine are reabsorbed by a common mecha-

nism since as the filtered load of glycine is increased creatine reabsorption decreases and is reduced to zero when the reabsorptive mechanism is saturated with glycine. On the other hand elevation of plasma creatine concentration had no significant effect on reabsorption of glycine. Thus the reabsorptive mechanism has a much higher affinity for glycine than for creatine. Since at sufficient loads alanine and glutamic acid also interfere with creatine reabsorption and since moderate elevations of plasma glycine significantly depress reabsorption of arginine Pitts subsequently [14] suggested that these four amino acids and creatine are reabsorbed by a common mechanism. It was also suggested that phosphate reabsorption shares a common element with alanine and glycine since elevation of plasma levels of the  $\alpha$  amino acids depresses phosphate reabsorption [15]. Beyer Wright Skeggs Russo and Shaner [16] were unable to confirm Pitts' finding that glycine depresses reabsorption of arginine.

Arginine and glycine may not be reabsorbed by a common mechanism in man. The aminoaciduria of cystinuria involves cystine lysine ornithine and arginine but not glycine [17] whereas certain aminoacidurias involving glycine do not involve arginine [18]. Indeed the recognition of glycinuria as an isolated reabsorptive defect was interpreted by DeVries and collaborators as evidence that glycine is handled by a tubular reabsorptive mechanism of unique specificity for this amino acid in man. While this may be correct an alternative explanation is that a single enzyme normally responsible for glycine alanine and creatine reabsorption (and perhaps of other substances) has selectively lost its ability to bind glycine through a minor structural alteration which is of no consequence for the binding of other substrates. Variation in a single amino acid of the peptide chain of the enzyme as in hemoglobins A S and C [19] might hypothetically have such a consequence. It would be of interest to know whether the glycinuric subjects of DeVries and collaborators also excreted excessive quantities of creatine.

#### *Renal Mechanism in Glycinuric Patients*

The reabsorptive defect in the glycinuric subjects was of impressive degree. The clearance of glycine approximated 70 ml per min in the index case a value perhaps fifteen times normal and larger than the glycine clearance reported by Levered [2] in patients with the adult Fanconi syndrome (41 ml per min) or Hartnup syndrome (29 ml per min) in whom a generalized aminoaciduria was present. In the subjects with glycinuria the excretion of free glycine ranged from 593 to 1 000 mg per day. In subjects with various oligo- or generalized aminoacidurias [2 13 19] the excretion of free glycine has ranged from 220 to 1 010 mg per day. Even the largest of these values approximates only 3 per cent of the daily glycine turnover in man. It is therefore not surprising that the plasma levels of glycine have been essentially normal in the glycinuric

subjects [1] and also in subjects with adult Fanconi and Hartnup syndromes [2] and Wilson's disease [19]

The relationship between excretion of glycine and formation of calcium oxalate stones in the glycinuric subjects remains obscure. Excessive conversion of glycine to oxalate seems quite improbable in view of normal urinary values of oxalate. The one stone which has been examined contained glycine which resisted simple elution with water but which was readily extractable in 30 min at room temperature in 1 *N* HCl. This finding should differentiate this type of oxalate stone from those commonly encountered in nonglycinuric subjects.

### GENETICS

Glycinuria is inherited as a dominant condition possibly sex limited, in the only available pedigree (Fig. 41-1). The pedigree is too small to

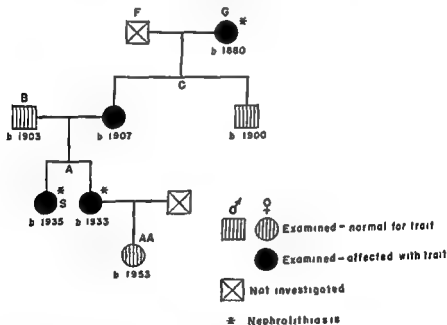


Fig. 41-1 Pedigree of glycinuria (Redrawn from Delvaux et al. [1])

establish sex limitation with certainty or to determine whether the trait is transmitted by an autosomal or sex chromosome.

### SUMMARY

1 Glycinuria is a rare disorder which has been recognized in four female members of three generations of a single family. It appears to be due to a selective renal tubular defect of glycine reabsorption.

2 Three of the glycinuric subjects had nephrolithiasis. The only stone analyzed was composed chiefly of calcium oxalate but it also contained a small amount of extractable glycine. The excretion of oxalate in the urine was normal.

3 The pedigree of the glycinuric family suggests that the disorder is transmitted as a dominant character possibly sex limited.

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## Chapter 42

### Cystinuria\*

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*W Eugene Knox*

✓ Cystinuria was one of the four diseases singled out by Garrod in 1908 [1] as the 'inborn errors of metabolism'. He postulated that cystinuria was due to a block in oxidation of cystine to sulfate. Increasing understanding of this disease has necessitated revision of Garrod's original concept and expansion of the 'inborn errors' to include not only hereditary defects of dissimilative and synthesizing enzymes but also defects of certain other functional systems such as renal tubular transport mechanisms.

✓ Cystinuria is now known to be a hereditary anomaly of renal function in which there is impaired renal tubular reabsorption of cystine, lysine, arginine, and ornithine. These amino acids, two of them essential to the body, are excreted in the urine in abnormal amounts throughout life. Only the excretion of the least soluble one, cystine, was recognized until recent times, and both the name and the clinical importance of the condition are entirely referable to this one amino acid. The only clinical consequence appears to be the frequent formation of urinary calculi composed of almost pure cystine. Some of the individuals heterozygous for the trait of cystinuria also excrete more than the normal amount of cystine, but the amount is not large, and these individuals only rarely form stones.

✓ Few of the aspects of cystinuria now remain unknown. Perhaps the major deficiency is proof that the two hereditary types, the recessive and incompletely recessive forms of the disease, are caused by allelic genes. But much remains to be done on the practical side in the recognition and treatment of cystinuric patients. The principles are well established and if effectively utilized could probably prevent calculus formation and its complications in most homozygotes.

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There is much to be learned from the multitudinous research efforts on cystinuria in the past. The approach to be employed in this chapter is historical in order that from a vantage point of contemporary concepts the tortuous development of knowledge of the disease can be viewed in illuminating perspective.

## EARLY HISTORY AND DEFINITION

### *Initial Recognition*

Cystine and cystine stones were recognized long before cystinuria. Indeed the cystine with which many studies began was provided by the



Fig 42.1 Cystine tones from bladder (left 50.2 gm) from kidney (middle) and from ureter (right 40.7 gm) of patients originally reported by Vornor [5]. Although their appearance may be distinctive many such stones cannot be identified without chemical analysis (By permission of A. Renand, [6]).

patients themselves upon whom the supply of this essential material was dependent for nearly 100 years. In 1904 [2] and even in 1936 [8] cystine metabolism was tested by giving to an individual cystine from a stone which he had formed.

Wollaston, who 13 years earlier had described five different types of urinary calculi and demonstrated that extrusions from gouty tophi were identical with one of these, added a sixth type in a report to the Royal Society of London in 1810. On Cystic Oxide, a New Species of Urinary Calculus [4] (see Fig 42.1). Two examples of bladder stones had come into his hands. Wollaston considered the substance to be an oxide because of its tendency to unite with both acids and alkalis. Because of its occurrence in the bladder (Greek *kystis*) he gave it the name *cystic oxide*.

The name became a focus in the first debates about the new disease. Stones do develop in the bladder in cystinuria [5] especially in young

people. It was assumed that cystine stones were, therefore, formed by the bladder until in 1818 Marcet found three patients (two at autopsy) with renal stones of cystine. Berzelius observed that the substance was not an oxide and suggested the name *cystin*. Garrod observed in his third Croonian lecture [1] of this new name which has since been universally adopted. Civiale wrote in 1838, that although it corrected an error of chemistry, it perpetuated an error of physiology, for cystine is excreted by the kidneys and does not have its origin in the bladder.

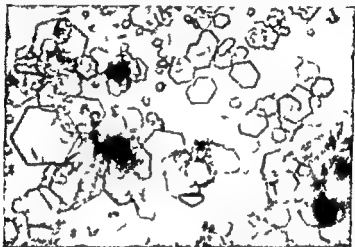


Fig. 42-2 Typical microscopic hexagonal plates of cystine in sediment of cystinuric urine. Angles are each very nearly  $120^\circ$  despite irregular shape. (By permission of J. Renander [5].)

The renal origin of cystine was established when Prout in 1820 and Strohmeyer in 1824 (cited in [3]) recognized the same hexagonal platelets of cystine in the urinary sediment which Wollaston had formed from dissolved stones. The identification of these crystals through a microscope has remained until recent times the primary means of diagnosis of cystinuria (Fig. 42-2). The collection and weighing of these crystals was first done in 1855 by Toel (cited in [5]) who found a cystine excretion of 1.33 to 1.50 gm per day in a cystinuric patient. This procedure also became the basic method for the study of cystine metabolism until it was replaced after 1900 by Folin's urinary sulfur fractionations.

The slowly accumulating information from sporadic cases of cystinuria was harvested and first given form by Niemann in 1876 [6]. He tabulated in chronologic order and discussed in addition to his own patient, 52 cases which he found in the medical literature.

### *Diagnostic Criteria*

Nearly all the cases in Niemann's collection had been diagnosed on the basis of urinary stones of cystine although several had been recognized

by cystine crystalluria. He made careful drawings and crystallographic studies of these transparent shiny hexagonal leaflets seen with a microscope in the sediment of urine which had been allowed to stand for a few hours (Fig 42-3). The identification of cystine rested primarily upon

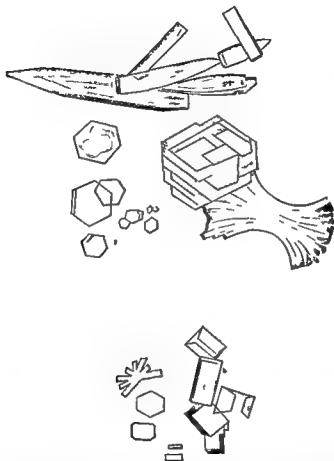


Fig 42-3 Niemann's engravings of crystal forms of cystine. Only microscopic hexagonal plates form spontaneously in urine (middle left). Recrystallization by evaporation after solution with ammonia gives plates and prisms or prismatic needles grouped above and to right of hexagonal plates. Recrystallization from acetic acid (below) rarely gives hexagonal plates but numerous prismatic forms. (From Niemann [6].)

Wollaston's criteria: the solubility of the crystals in ammonia and in solubility in acetic acid and when strong HCl was added to the dry crystals under the microscope the rapid growth of stellate clusters of delicate prisms consisting of the water-soluble cystine hydrochloride. Chemical analysis depended largely upon the "sulfur reaction": the

formation of a precipitate of lead sulfide when urine or a cystine solution was heated with KOH in the presence of lead acetate. He recognized that cystine could be present in the urine in abnormal amounts as indicated by the lead sulfide precipitate without the presence of stone or crystals.

By Niemann's criteria, a diagnosis of cystinuria suggested by a stone or crystals was confirmed by the identification of cystine through the strong sulfur reaction given by the solid material. Additional confirmatory evidence was the smell of hydrogen sulfide when the urine was allowed to stand long in hot weather, or the foul smell due to cadaverine and putrescine formed by bacterial action on the other amino acids now known to be present in these urines in abnormal amounts. Since the methods for detecting cystine depended upon precipitation from supersaturated solutions, it is not surprising that Niemann was cautious about excluding the diagnosis of cystinuria. He noted that cystine crystals are sometimes absent from the urine of individuals with a known cystine stone, that cystine crystalluria is sometimes present without stone, and that sometimes stones of other chemical composition are found in known cystinuric patients. The latter are now known to develop because of the urinary infections set up by the original stones.

### *Patterns of Occurrence*

Both males and females are affected with cystinuria, and males predominate. Niemann's collection of 53 patients was comprised of 38 males, 14 females, and 1 patient of unstated sex. A similar predominance of males, though slightly less marked, has characterized all subsequent collections of cases [7-9]. No significant sex difference, however, occurred in a later group of cases diagnosed by chemical examination [10].

Niemann believed that cystinuria is present from birth. The youngest patient seen up to that time was 2 years old. Frequencies of stone formation were similar in the successive decades of life [6], and no stones were reported after age 50. Since then a cystine stone has been passed spontaneously by a child of 9 months [11], and several cases are now on record of stone formation in older persons, including a woman of 87 [12]. In one patient stone formation occurred at age 42 and recurred at age 81 [13].

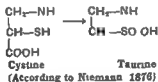
Niemann commented on the familial disposition of cystinuria. The fourth and fifth cases of Marcet (cited by Niemann) in 1818 were two brothers about 30 years old. Four other instances in which two siblings were affected had been reported, and in one of these the mother had also excreted abnormal amounts of cystine but did not have stones or crystalluria. It is interesting that the brother of Niemann's patient, who might well have had the same genotype, excreted abnormal amounts of cystine by chemical test; the mother and maternal uncle of the patient were normal in this regard.

### Possible Causes

His large collection of cases put Niemann on firm ground to evaluate the alleged constitutional or environmental causes of the disease. There was no association with occupation, social standing, housing, or nutrition or with scrofula, anemia, or rheumatism.

In 1868 Marowsky [14] from observations on a patient with cystinuria who also had chronic acholia and liver disease theorized that cystinuria is caused by liver disease. He believed that in cystinuria the usual taurine-secreting activity of the liver is suppressed and replaced by vicarious secretion of the sulfur-containing taurine through the kidney in the form of cystine. Niemann, however, found no evidence for the appearance in the urine of other fragments of the bile acids, and Lewis [16] later cited analyses showing no deficiency of taurocholic acid in the bile of cystinuric patients.

Niemann revamped Marowsky's hypothesis into the form later to be known as an arrest of metabolism—a concept from which Garrod developed his fertile idea of the inborn errors of metabolism. Instead of taurine's being converted to cystine as Marowsky implied, the formulas of the substance as then known



indicated to Niemann the reverse relation (the correct formulation does not alter this conclusion) that taurine is the natural oxidation product of cystine. His own measurements disclosed a low sulfate excretion in cystinuria. He failed to add the now obvious suggestion that a failure of cystine oxidation would cause the low urinary sulfate, but the pathway of cystine oxidation through taurine to sulfate was not yet known.

### Treatment

No treatment was used for Niemann's patient [6]. He reviewed earlier attempts at treatment and suggested that alkalinization of the urine might dissolve the cystine. Magendie is credited with the same suggestion, plus restriction of protein (cited in [5]). On one occasion Niemann's patient excreted an alkaline urine containing cystine crystals. This observation, in addition to the fear of precipitating phosphate with alkali and so enlarging the stones already present, restrained him from trying alkali therapy. Only surgical treatment was offered when necessary, and that sometimes tardily by modern standards.

### *Definitions*

Niemann's success in defining cystinuria, achieved largely by the use of logic to divest the known cases of their adventitious trappings, can be measured by comparing the conclusions with the definition of cystinuria written in 1955 by Dent and Senior [16]

It is a condition presumed to be present from birth and characterized by the excretion in the urine of large quantities of cystine (in the adult about 1 gm in 24 hours) lysine arginine and ornithine. The condition is often present in the patient's siblings and may result in the formation of stones composed almost entirely of cystine. Apart from the possibility of kidney damage and the other complications resulting from stone formation, the patients enjoy good health and are clinically indistinguishable from normal.

Niemann's definition agrees with this modern one in every detail, with the exception only of the abnormal amounts of lysine, arginine, and ornithine in the urine of these patients.

## CLINICAL AND BIOCHEMICAL NATURE OF CYSTINURIA

### *Clinical Reports*

After Niemann's review little could be added of clinical importance except validation of his definition of the disease until additional understanding was available. This was long in coming. Ebstein [17] 8 years later added 10 cases, and in 1900 Simon [18] brought the total to 103 cases and added 4 of his own and 1 from the Norwegian literature. One of his cases was a Negro, the only one reported to have cystinuria. These cases and others have been repeatedly reviewed [7, 19-21]. Sum Schick counted 180 cases by 1929 [22]. In the 10-year period between 1921 and 1932 Lewis [16] counted 71 newly reported cases. Morrison [11] listed 75 cases studied radiographically between 1920 and 1940. Renander's estimate that published cases by 1940 only slightly exceeded 200 was conservative [2].

Some cases have been of unusual interest. Southam [23] removed a cystine stone from a woman who 14 years postoperatively still excreted cystine. The mother of this patient had been operated on 24 years earlier by Southam's father also for cystine stone and she continued to excrete cystine for 8 years. The chronicity of the disease was further documented by a metabolic study in 1923 by Looney, Berglund, and Graves [24] of a patient studied 17 years earlier by Alsberg and Folin [25].

A dignified rivalry to report the largest stone can be discerned in the clinical literature. Chaos governed the contest since the participants frequently confused weights in grams with grains. The record, attested by an independent weighing 27 years later when the stone reposed in the Museum of the Royal College of Surgeons in England, was long held

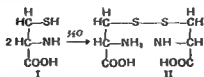
by a vesical calculus weighing 68.04 gm and described by Harrison in 1849 [26] Lank found a stone of 86 gm in 1912 [21] Ancherson in 1914 removed from the bladder of a Norwegian male a cystine stone weighing 102.5 gm the largest that has ever been reported [27]

### Confusion with Cystinosis

A most notable confusion about the clinical nature of cystinuria was introduced by Abderhalden's description in 1903 of another disease of cystine metabolism [28]. Although Abderhalden called the new disease *familial cystine diathesis* the majority of medical writers up to the present time have considered it to be a manifestation of the same hereditary disturbance as cystinuria. After the next cases were described in 1926 by Lignac Leiden [29] it was also called *cystinosis* *cystine storage disease* and *Lignac-de Toni Fanconi syndrome* (see Chap 37). The first patient was seen only after death at age 21 months. The internal organs were potted with crystalline deposits of cystine. Abderhalden demonstrated abnormal amounts of cystine in the urine of two siblings the father (9.2 mg per 100 ml) and the grandfather (14.0 mg per 100 ml) of his patient. A reason for additional confusion was that Lignac's first case had ureteral and renal cystine stones (1926) a unique finding in any condition except cystinuria. The typical findings of cystinosis all of which distinguish it from cystinuria are retarded growth and development, rickets and osteomalacia, low serum phosphorus, glycosuria, generalized aminoaciduria of 10 or more amino acids among which cystine is usually not prominent, very rare formation of cystine stones, the presence of cystine crystals in the tissues of children but not adults with the disease [30] and its frequently fatal termination.

### BIOCHEMISTRY OF CYSTINE

A more precise definition of cystinuria was dependent upon knowledge of the chemical nature of cystine and of its metabolism. It would seem that both these aspects were well on the way to solution by 1900. Morner in 1899 [31] had discovered cystine in protein and found hydrolyzed hair to be an unusually rich source. The correct chemical formulas of cystine (II) and its reduced form cysteine (I) were established in 1902 by Friedman [32]



Chemical methods to measure urinary neutral sulfur (largely cystine) and organic sulfate were developed. The first balance studies on the sulfur

metabolism of a cystinuric person were reported in 1903 by Alsberg and Folin [2a]

Loewy and Neuberg [2] reported in 1904 that hair and stone cystine are metabolized differently and are different chemical substances. Alsberg and Folin [2a] promptly reported their own and other evidence against this conclusion. They were unable to verify such a difference (which hinged on a difference of crystal form), but as late as 1927 after the analysis of the 78 gm of stones from Tennant's patient [33] this error was repeated [34]. There was further confusion when it was observed that an increase in food protein produced an increase of cystine in the urine but when the food protein was hydrolyzed outside the body and the isolated cystine was given to the patient, that cystine was oxidized to sulfate. Cystine itself even cystine isolated from the patient's own urine [3a] caused no extra cystine to be excreted. Garrod [30] expressed the pessimism of the time when he wrote

The study of the metabolic peculiarities of cystinurics has yielded results which are very difficult of explanation and the more the problem is investigated the more remote seem to be the chances of its satisfactory elucidation. Thus in every case tested except that of Loewy and Neuberg in which cystine has been given by mouth it has been completely destroyed although the patients were all the time excreting cystine as such.

During the same period other observations were made from which the modern understanding of cystinuria could have been deduced. But like a hank of string with the key loop in sight, several pulls at other loops resulted in a snarl which took nearly 50 years to untangle.

#### CYSTINURIA AS AN 'INBORN ERROR OF METABOLISM'

Garrod announced his brilliant concept of the inborn errors of metabolism at the height of the confusion about cystine metabolism [1]. According to Garrod's thesis a hereditary condition characterized by the accumulation of an abnormal metabolite resulted from the hereditary lack of an enzyme that normally removes that metabolite. Here was an illuminating way to regard the accumulation of cystine, which in a normal individual is oxidized to sulfate. Cystinuria was included as one of the inborn errors of metabolism. This helped to obscure for a generation that a supposedly degradative enzyme in cystinuric subjects was in fact not missing. In both normal and cystinuric subjects cystine administered orally as such was oxidized and excreted as sulfate. The defect in cystinuria was manifested only if some precursor of cystine such as protein was fed. Garrod was not unaware of this paradox but he contented himself with the explanation that the metabolic error was partial and incomplete and that the contradiction would be resolved when more was known of the intermediary steps of sulfur metabolism.



There was ample evidence of the hereditary nature of cystinuria. Pfeiffer [37] reported four cystinuric children of parents who were first cousins. Cohn [38] described a family with a cystinuric mother, a normal father and 10 children. Six of the children were cystinuric, 2 definitely not and 2 untested. Two of the children were twins, both cystinuric. Identical twins, both with the disease, who also developed stones almost simultaneously, were later described by Kretschmer [7] and in another instance by Harris and Warren [39]. Garrod saw that this pattern was *meaningful*: homozygotes for a rare recessive gene would usually occur among siblings, rarely in other generations of the same family, and more commonly in families where a consanguineous marriage improved the chances that both parents would possess the rare gene. He did note, however, that there was a greater frequency of direct transmission of cystinuria from parent to child than was met in connection with the other metabolic errors he described (i.e. albinism, alcaptonuria and pentosuria). Fifty years later the recognition of a dominant form manifesting cystinuria in the heterozygote confirmed his impression [10].

#### METABOLISM OF CYSTINE

In 1935 the biochemist Brand and the urologist Cahill, with two young cystinuric patients, began to elucidate the metabolic pathway of the sulfur-containing amino acids by feeding experiments. The erroneous assumption of a block in cystine metabolism was useful. The identification of cystine precursors was as well served by the renal leakage of the extra cystine as it would have been by the supposed metabolic block. The new sulfur-containing amino acid, methionine, was promptly identified as a cystine precursor [40]. Proteins with high methionine content produced greater cystine excretion than proteins with low methionine content. The cystine in the protein was oxidized to sulfate [41]. This identification of methionine, and not cystine, as the principal dietary source of urinary cystine was confirmed by Lewis [3] and partially resolved the paradox described earlier. The subjects of Brand's experiments formed extra cystine from cysteine and from homocysteine but not from cystine or homocystine [42]. Brand postulated that cysteine is a product of the catabolism of methionine and that the error in cystinuria is a failure of the proper utilization of cysteine, not a failure of cystine metabolism. The final results clearly indicated a pathway of metabolism of sulfur-containing amino acids with the surprising sequence of reactions shown in Fig. 42-4 [40].

The excretion of cysteine as cystine by cystinuric patients and oxidation of cystine by both normal and cystinuric persons was in accord with what was known. The reaction, difficult to believe, was the shortening of the carbon chain in the conversion of the  $\gamma$  thio compound, homocysteine, to the  $\beta$  thio compound, cysteine. Lewis confirmed the essential

findings for the scheme of Fig 42-4, but he refused to accept the same conclusion

Brand's scheme [43] was confirmed in detail by studies from du Vig neaud's laboratory  $S^{35}$  labeled methionine gave rise to labeled cystine but  $C^{14}$ -labeled methionine did not [44] The shortening of the carbon chain occurs by transfer of the sulfur from the four-carbon compound homocysteine, to a different three carbon compound, serine, to form cysteine made up of the original sulfur but with new carbon atoms The intermediate of this interchange is cystathionine, a combination of both homocysteine and serine in one molecule This compound, too was later shown to give rise to cystine [45]

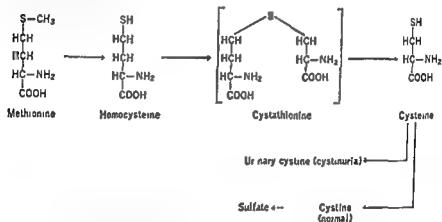


Fig 42-4 Pathway of metabolism of sulfur amino acids established in cystinuric patients by feeding experiments (From Brand et al [40]) Cystathionine was discovered later

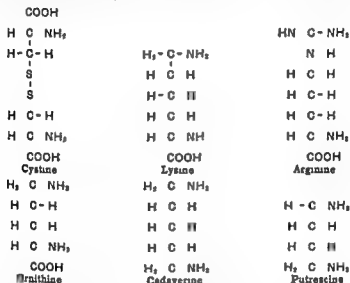
After the pathway of sulfur amino acid metabolism in man was delineated by Brand through his studies of a patient with cystinuria a similar pathway (in reverse direction leading to methionine) was proved for *Neurospora* by the use of several mutants of this species [46]

The studies of Brand and his group still fell short of a full explanation of the disease The enzymic apparatus for cystine degradation by the cystinuric patient is obviously intact since cystine given as such is excreted as sulfate Any amount given orally can be oxidized by the liver but if cystine or cysteine is given parenterally to dogs or rabbits [47] or to a cystinuric patient [48] it is excreted by the kidney as cystine Failure of tubular resorption was later implicated quite directly by the fact that the level of cystine in the blood of cystinuric patients is not elevated [49] This eliminated the possibility of a renal overflow mechanism although it was apparently not appreciated by the authors at the time The meaning was not lost to all however for in 1934 Rabinowitch suggested that the mechanism of cystinuria is analogous to that of glycosuria

in renal glycosuria (which he thought was a result of an undue 'permeability' of the kidney [50]) There was left unexplained then only why the kidneys are presented with a greater load of cystine for excretion when a precursor is fed than when cystine is fed as such this question has still not been answered [51]

### AMINOACIDURIA OF CISTINURIA

An earlier series of experiments might have led to the recognition of the other amino acids (lysine arginine and ornithine) that are present in abnormal amounts in cystinuric urine



Ellinger [52] in 1898 found that bacteria would decarboxylate lysine and ornithine to form the foul smelling ptomaines or diamines cadaverine and putrescine. These substances had been isolated from the urine of many [18, 20] but not all cystinuric patients. The original patient studied in 1889 [53, 54] had cystitis and successive investigators over several years found the amounts of diamines increasing in pace with her urinary infection [55]. Recently Harris et al [10] reported a cystinuric patient who was exceptional among their patients in excreting no lysine. Upon closer examination they found a urinary tract infection and further analysis of the urine revealed the missing lysine present as cadaverine.

It seems now almost inconceivable that the work of Alsberg and Folin in 1905 [25] and its extension by Wolf and Shaffer in 1908 [48] did not quickly solve the problem of cystinuria. Alsberg and Folin tested the theory of defective hydrolysis of dietary protein by measuring the 'undetermined nitrogen' fraction of urine i.e. that part of the total

findings for the scheme of Fig 42-4, but he refused to accept the same conclusion

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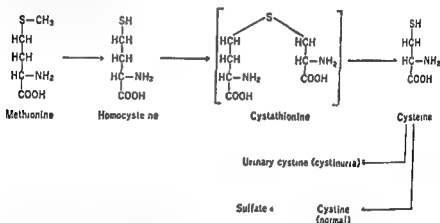


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The project languished and the dogs were dispersed sometime before Brand's death in 1953, and before the new concept of the renal tubular mechanism in human cystinuria could be tested on cystinuric dogs. Metabolic studies nearly duplicated the findings from contemporaneous human studies. Cystine accounted for 13 to 17 per cent of the total urinary sulfur in the cystinuric dogs [62-64] compared with about 20

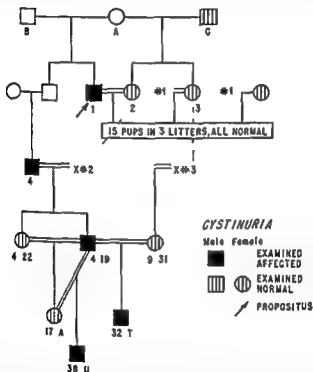


Fig 42-5 Known pedigree information of cystinuria in the Herrers. Original breeding system was retained. Litters were undoubtedly larger. A total of more than 300 descendants of dogs 1 and 4 were tested and 1 cystinuric animal, all males, was found. (From Brand et al [62] and W E Hess and M A Sullivan [63].)

to 30 per cent in the human being with cystinuria [3]. Extra cystine was excreted when extra protein, methionine or cysteine was fed. The feeding of cystine did not result in extra cystine excretion [65].

It remains to be shown whether the same discrete renal transport system under hereditary control is affected in the cystinuria of both men and dogs. It was in normal dogs that the competition between arginine and lysine for renal tubular excretion was first demonstrated [66]. This finding was initially the basis for the belief that the excretion of the several amino acids in man could be due to a deficiency of a single renal

nitrogen left after subtraction of that determined independently in urea ammonia creatinine, uric acid and cystine. Their patient excreted abnormally large amounts of the undetermined nitrogenous substances as well as cystine during starvation. They concluded that these were produced by 'endogenous metabolism' but both fractions were also increased in proportion to the protein in the diet and therefore were also produced by 'exogenous metabolism'.

Four years later Wolf and Shaffer [48] confirmed the results of Alsberg and Iolin in detail and emphasized that there is an abnormal excretion of 'undetermined nitrogen' in cystinuria and that this is at least in part due to other amino acids. Somehow, this was forgotten. Later isolations of crystalline lysine derivatives from cystinuric urine by Ackermann and Kutscher in 1911 [50a] and by Hoppe-Seyler in 1933 [50] passed almost unnoticed and the observations were not corroborated. Kutscher obtained amounts equivalent to 0.1 gm lysine per liter of urine which was grossly more than could be present in normal urine. The discovery and identification of three other amino acids besides cystine in cystinuric urine 40 years later was a major forward step in the understanding of this disease.

#### CYSTINURIA IN ANIMALS

The sporadic occurrence of urinary cystine stones in dogs was reported in 1823 by Iassaigne [57] in 1861 by Gross [58] and by Lentz in a male dachshund in 1921 [59]. The stone was always recognized after the death of the animal and no metabolic studies were made. The tendency to form cystine stones was not associated with a single breed, as is the frequent development of urate stones in the Dalmatian coach hound. In 1935 Morris [60] found that an Irish terrier suffering from urinary retention had cystine crystalluria. At operation several vesicular and urethral calculi of cystine were successfully removed [60]. Brand and Cahill joined the study, made the necessary chemical measurements to identify the disease and arranged a breeding program to investigate the genetics of the condition.

Through the American Kennel Club registrations of the sire and dam of the first cystinuric dog, four related dogs were found to begin a breeding program. One, a cousin of the proband, also had cystinuria [61]. Twenty-five unrelated animals of the same breed were not cystinuric. Three hundred dogs of the cystinuric strain were raised and 12 cystinuric animals were identified. All affected animals were males [61, 62]. The original cystinuric male produced litters with an unrelated female and with two half sisters. None of the 15 pups was cystinuric [64]. No genetic analysis of the results was ever made, but the few published pedigrees resembled, or at least did not exclude, sex-linked inheritance [62, 63], one quite different from the recessive pattern seen in man (Fig. 42.5).

excretions about ten times the normal level [76] Equally powerful and even more convenient methods of amino acid analysis soon confirmed these new abnormalities Column chromatography of the urinary amino acids in six cystinuric patients all with histories of cystine stone formation showed that a definite pattern of aminoaciduria was characteristic of this disease [77] In addition to the cystine lysine and arginine already identified ornithine was also found in abnormal amounts The amounts of the remaining amino acids were not different from normal

The first explanation of the large amounts of new amino acids found in the urine of cystinuria was progressive renal damage producing first the leakage of these few amino acids and later perhaps the generalized aminoaciduria seen in the Fanconi syndrome [76] However long clinical experience belied this A nonspecific renal damage would not show such specific leakages

### *Renal Physiology and Aminoaciduria*

The possibility that a discrete renal tubular transport mechanism with enzyme like specificity could be defective in cystinuria and permit the specific aminoaciduria was first appreciated in 1951 It was known that arginine and lysine compete for the same tubular reabsorptive mechanism in the kidney of the dog each lowering the  $T_m$  of the other [60] Dent and Rose [78] suggested that the reabsorptive mechanism was missing in cystinuria The first evidence to substantiate this was an elaboration of an old finding by Brown and Lewis in 1937 [49] that the concentration of cystine in the blood of a cystinuric patient is not elevated In 10 normal and 9 cystinuric individuals the concentration of cystine in the plasma averaged 0.82 and 0.69 mg per 100 ml respectively [9] It was argued that if cystinuria was the result of a renal defect the plasma level would be normal or low as it appeared to be but that if it was the result of a blocked cystine metabolism the plasma level should be elevated Evidence was presented that the levels in blood are not above normal for the other amino acids also excreted in cystinuria [80] Stein and Moore [81] established that high plasma concentrations of amino acids could not account for the excretions since the levels of lysine and ornithine were normal and of cystine and arginine low in their patient

The crucial evidence for a specific renal mechanism was obtained from the renal clearance of the amino acids In a classic study by Dent Senior and Walshe [81] cystine clearance in normal individuals was of the order of 4 ml per min whereas in two cystinuric patients (both with considerable renal damage from stone formation and therefore probably with reduced glomerular filtration rates) the cystine clearances were approximately 100 ml per min i.e. they approached the glomerular filtration rate Moreover in a person known to be heterozygous for cystinuria, a definitely elevated cystine clearance (14 ml per min) was found

transport system. Opportunities to make observations on such dogs are apparently not rare. Krabbe [66] reported that 3 per cent of the urinary calculi removed from dogs in Holland and Denmark are cystine. In England they accounted for 18 per cent, according to White [67] and were the stones which most commonly caused urethral impaction. Crane and Turner [68] found cystine and lysine predominating in the urine of a Labrador retriever who had two episodes of cystine vesical calculi with urethral impaction. No abnormal quantities of arginine or ornithine were found. Study of the plasma was inconclusive. This is the urinary pattern expected in the heterozygous form of incompletely recessive cystinuria in man (see below).

A second species of animal with cystinuria is the blotched genet, a wild cat from Kenya. In a survey by paper chromatography of the amino acids excreted by different animals at a zoo, Datta and Harris [69] found that every blotched genet was cystinuric. The condition was present in a number of unrelated members of the species, so it was not just a familial condition *within* the species, but a species characteristic.

The cystinuria of the Kenya genet differs from that in man in that high concentrations of other amino acids are not excreted. Dent and Senior [16] also established that the cystinuria occurred by a renal mechanism, i.e., a deficiency of tubular reabsorption. Strangely enough, the concentration of cystine in the genet urine is 1 to 3 mg per ml *in true solution*, that is, about four times its solubility in human urine. Yet the genets do not form urinary cystine stones. The failure of cystine to precipitate from these urines raises again the possibility of a cystine-containing complex existing in cystinuric urines of man [40, 70, 71] and dog [63], although the existence of such a complex has been emphatically denied [3]. No additional cystine, lysine, or arginine is found in human cystinuric urine after hydrolysis [72]. The early belief in such a cystine complex may have arisen from a theory that when cystine is absorbed as a peptide because of faulty digestion, it escapes oxidation and produces cystinuria [73]. No stable complex of cystine was found in genet urine. The unusually high urea concentration of 10 to 20 gm per 100 ml in genet urine, and the presence of other salts and amino acids [74] might affect the solubility of cystine.

Recently cystine urinary stones have been discovered in a third animal species, the mink [70].

#### RENAL TIBULE AND CYSTINURIA

##### *Pattern of Aminoaciduria*

In 1947 microbiologic determinations of the individual amino acids in the urine of a cystinuric girl (compared with analyses in seven female controls) showed a high cystine excretion, but also arginine and lysine



excretions about ten times the normal level [76]. Equally powerful and even more convenient methods of amino acid analysis soon confirmed these new abnormalities. Column chromatography of the urinary amino acids in six cystinuric patients, all with histories of cystine stone formation showed that a definite pattern of aminoaciduria was characteristic of this disease [77]. In addition to the cystine lysine and arginine already identified ornithine was also found in abnormal amounts. The amounts of the remaining amino acids were not different from normal.

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Elevated renal clearances in cystinuric patients of cystine, arginine, ornithine, and lysine were then demonstrated by Robson and Rose [82]. The clearances of cystine, arginine, and ornithine were not further increased by the rapid intravenous infusion of 5 gm of L-lysine, because the clearances were already near the glomerular filtration rate. In normal individuals and heterozygotes lysine caused transient increases in the clearances of the three other amino acids, and only of the three that are excreted in cystinuria. This is analogous to competitive inhibition of an enzyme.

Doolan, Harper, Hutchin, and Alpen [72] also established that the plasma level of lysine was lower than that of normal subjects in four cystinuric patients (2.4 compared with 3.2 mg per 100 ml) and that extra lysine (and arginine) was cleared at a rate similar to that of inulin, i.e. without reabsorption. The excretions observed were much too large for leakage of filtrate past a few inactive nephrons. A specific defect in renal tubular reabsorption was therefore established.

Cystinuria may then be defined as a hereditary inactivity of a specific renal tubular transport system which normally combines with and reabsorbs the several related dibasic amino acids. The inactivity could result from an absence or defect of a specific enzyme-like combining substance in the tubular cells. It has been suggested that the enzyme-like combining substance of the transport system fits substrates with two suitably separated amino groups, and that this is the structural basis for the common transport of these four dibasic amino acids (see Effect of Genotype on Renal Tubular Transport below). Within the group the affinity for the combining substance ( $K_m$  of an enzyme) is less for cystine and lysine than for arginine and ornithine, as the genetic studies have indicated (see below).

With the demonstration that a specific renal transport system for certain amino acids is lacking in cystinuria, it became possible to ask if this same transport system is operative in other tissues besides the kidney. The absorption of amino acids by the intestine and their concentration into cells, for example, occurs with transport systems apparently similar to those of the kidney tubule. There is no positive indication of interference with such systems in cystinuria except in the kidney. Only direct tests could rule out such a possibility, however, and one such observation has been recorded. Leukocytes of normal and cystinuric individuals take up cystine and lysine equally efficiently. There is no mutual inhibition of uptake by the cells between these two amino acids [83].

#### INCIDENCE OF CHEMICAL AND CLINICAL CYSTINURIA

Most surveys [22-84] of which Morner's is the most complete [85] have generally agreed that about 1 per cent of all urinary calculi are

cystine There are no reliable estimates of the incidence of urinary calculi in the general population with which the absolute incidence of cystinuria can be correlated

The incidence of cystine crystalluria has been reported to be 1 case in 15 000 [18] one in 20 000 [86] and four in 35 000 [87] The over all incidence is therefore approximately one in 10 000 urine specimens This is possibly higher than in the general population since undoubtedly only selected (e.g. hospitalized) populations were tested

The apparent incidence of cystinuria depends largely on the prevailing interest in the disease Morner's experience in Sweden illustrates this The first case was seen in Sweden in 1870 and the second in 1901 Morner [85] reported 6 seen during the period 1901 to 1920 Sixteen additional cases only 4 without stones were observed in the next 5 years [9] The diagnostic criteria had not changed from the earliest time By 1936 Morner had found a total of 36 cases of cystinuria in the generation of the Swedish population living since 1901 Renander [6] then surveyed independently all hospitals in the country and succeeded in finding only one additional case These studies approach a complete ascertainment of cystinuria in a mean population of 6 million and give a (probably low) incidence of roughly 1/200 000

Between 1929 and 1931 Lewis examined the urine of 10 534 University of Michigan students with the cyanide nitroprusside test and when that was positive by the more specific Sullivan test for cystine [8] The normal excretion of cystine is in the range of 40 to 80 mg per day [88] and approximately the same amount per gram of urinary creatinine [39] It has been estimated that Lewis's cyanide nitroprusside test would be distinctly positive with at least 100 to 125 mg cystine per gram of creatinine [39] Lewis discovered 18 individuals or 1 in 600 who consistently excreted cystine Eleven of these were studied in more detail and all showed an abnormally elevated urinary organic sulfur fraction Four showed repeated cystine crystalluria (4 in 10 000) Earlier estimates of the incidence of cystine crystalluria were less than one fourth this high but they were based on single examinations for a condition known to occur only intermittently None of Lewis's subjects had ever had symptoms suggestive of renal stone or colic and only one gave a family history of such disease A sister of one of the individuals had formed a cystine stone but his parents and five other siblings did not excrete cystine The urine of 22 other individuals in the population studied gave weakly positive tests consistently or occasionally The total incidence of positive tests was 1 in 250 Those giving weakly positive tests had nearly normal sulfur partitions and were considered normal Malleon (cited in [39]) confirmed the high incidence of chemical cystinuria (1 in 250) with the finding of four positive urines from approximately 1000 students at University College London These urines contained 106 to 161 mg

cystine per gram of creatinine, and paper chromatography demonstrated in all an increased output of lysine as well as cystine

The finding of excess excretion of cystine by 1 in 250 people introduced a new entity, chemical cystinuria. An additional criterion besides cystine excretion was obviously necessary to identify the specific disease cystinuria in stoneless persons, and to distinguish it from other conditions which might cause chemical cystinuria. The specific aminoaciduria with four amino acids provided the criterion in the absence of stone formation. The high frequency of chemical cystinuria found an explanation in the frequency of heterozygotes that can be deduced from studies of the genetics of the disease as determined from the specific aminoaciduria.

Dent and Harris undertook a study of cystinuria in terms of the specific aminoaciduria [89]. The result was simply to redefine what has been called cystinuria as a specific hereditary condition characterized by the presence of the four amino acids in abnormal amounts and to prove that it is distinct from the inherited Fanconi syndrome and from Wilson's disease. Studies of the families of three individuals with the latter two diseases disclosed three other individuals with a general aminoaciduria and none with the specific cystine-arginine-lysine-ornithine type of aminoaciduria. The families of seven classical cystinuric patients, six of whom had stones, contained seven more individuals of the same specific type and none with generalized aminoaciduria. These findings directly disproved the idea that cystinuria is a stage in the development of the Fanconi syndrome [76].

#### PATTERNS OF INHERITANCE

The familial incidence of cystinuria among siblings was such that Garrod suggested its inheritance as a rare recessive trait. Even then, however, too many instances of direct transmission from parent to child were known and Garrod entertained the possibility that the condition might be dominant. An alternative explanation, that the gene is very common instead of rare, would also account for the presence of the disease in two successive generations. This explanation gained credence when it was known that chemical cystinuria is much more common than the incidence of stone formation.

From a consideration of the published pedigrees of cystinuria, complete except for those of Niemann [6], Kretschmer [7] and Morner [9, 90], Dent and Harris concluded that the available data were of little genetic value since only five families had been examined with chemical methods and in none of these was there information about the excretion of other amino acids. The data from the earlier studies simply reemphasized the mutually contradictory points made by Garrod: the frequency of parental consanguinity and of cases among siblings indicates that cystinuria is a rare homozygous recessive disease, whereas the presence of affected

individuals in three successive generations of the same family [91] almost certainly indicates that some patients are heterozygotes i.e. that the condition is sometimes dominant.

Analysis of the new family data collected by Dent and Harris did not resolve this problem. In two instances the parents of cystinuric patients were normal, so that the condition is not regularly manifested in heterozygotes. The occurrence of affected parent-child pairs in two of the seven families could be explained on the recessive hypothesis with a postulated high frequency of the gene in the population (one individual in 12 would be a heterozygote if Lewis's incidence of 1 in 600 represented the homozygous form). But if the gene is so common consanguinity would not be important for its expression. Yet even in this small series there was one consanguineous marriage. It was apparent that the genetics of cystinuria, half known for so long on the basis of the qualitative excretion of cystine, would yield fully only to a detailed quantitative study.

Quantitative studies by Harris and coworkers of the amino acid excretion in cystinuric families provided the answer. Measurements in 21 families [39] showed in some of the families the expected clear separation into cystinuric and normal individuals. In other families there was a continuous gradation from normal to extremely abnormal cystine excretion in the different individuals. It was evident that the disease appeared in two forms, separately inherited.

### *Recessive Cystinuria*

The first type, the more common of the two forms of cystinuria, appeared in families where all individuals excreted either normal or highly abnormal amounts of urinary cystine and no individuals had intermediary values. There was a sharp segregation for the property of cystine excretion and the family distribution was of the type expected if the cystinuria occurred in individuals homozygous for a rare recessive gene. The parents, who were apparently normal, were necessarily heterozygotes, but the one recessive abnormal gene which they possessed was not strong enough to create any noticeable effect on cystine excretion. Thus the defect appeared only among the homozygous offspring. Four instances of consanguinity in these families further supported this view. This type is called recessive cystinuria.

### *Incompletely Recessive Cystinuria*

In families of the second type, normal, intermediate and high values of cystine excretion were all found. The family distributions suggested that the individuals with moderately raised values of cystine excretion were heterozygotes. Those with high values, like the affected individuals in the recessive families, were homozygous for a rare gene. Because the character of the incompletely recessive gene in the second type of

family was strong enough to produce intermediary values in the heterozygotes, it was assumed that the individuals with normal cystine values did not possess the gene for cystinuria. Further quantitative studies of the other amino acids excreted made it clear that the earlier contradictory results had found their explanation in two different modes of inheritance of different degrees of the same renal defect.

### *Effect of Genotype on Renal Tubular Transport*

The variant forms of the disease originate in the inheritance of different degrees of the renal defect. This was clear from a study by Harris

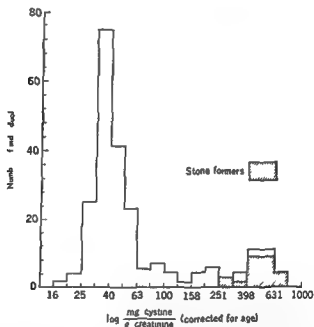


Fig. 42.6 Cystine output of cystinuric subjects and their relatives ranged from 15 to 800 mg per gm creatinine. Stone formers were clustered among the high cystine excretors. (By permission of H. Harris and F. L. Warren [32].)

and coworkers of lysine and arginine excretions [92]. Their patients included 28 stone-forming cystinuric persons and 121 of their relatives. All degrees of cystinuria from 20 to 800 mg cystine per gram creatinine were found. There was stone formation only in individuals who excreted 250 mg cystine per gram creatinine or more. This was the level at which a saturating concentration of cystine in urine would be likely to occur (Fig. 42.6). The lysine excretion was regularly about twice that of cystine and paralleled the cystine excretion throughout all its gradations. Arginine and ornithine were not excreted in abnormal amounts until the cystine and lysine reabsorptions were seriously impaired. Only with a

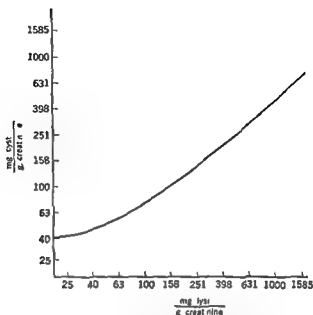


Fig 4-7 Cystine and lysine contents of urine from a series of cystinuric patients and their relatives (By permission of H Harris and E B Robson [94])

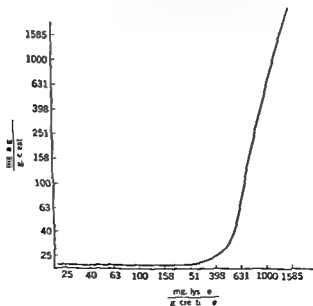


Fig 4-8 Lysine to cystine content of urine from a series of cystinuric patients and their relatives (By permission of H Harris and E B Robson [94])

cystine excretion of 250 mg per gm creatinine or more did arginine and ornithine also appear in the urine in abnormal amounts. The amounts then increased proportionally with the excretion of higher amounts of cystine and lysine. These relationships, which held in families with both recessive and incompletely recessive cystinuria, are shown graphically in Figs. 42.7 and 42.8. Subsequent studies have indicated that the variations in the arginine to lysine ratio are genetically determined in the recessive homozygotes and may indicate still other variants of the disease [93, 94].

These genetic studies revealed a hierarchy of affinities of substances for a renal transport system, comparable to the affinities of substrates for an enzyme. Arginine and ornithine had high affinities and were preferentially reabsorbed by the tubular mechanism. Cystine and lysine affinities were lower and their reabsorption failed first. The genetically determined degrees of the renal defect, if partial, permitted the preferential reabsorption of arginine and ornithine, and if more complete, resulted in the renal loss of all four amino acids including cystine, in sufficient concentration for the latter to precipitate and form stones.

#### *Phenotypes and Genotypes and Incidence of Cystinuria*

The biochemical and genetic data of Harris et al. [39-92] were finally correlated in a definitive paper, 'Phenotypes and Genotypes in Cystinuria' [10]. A total of 29 families, each with at least one stone-forming cystinuric member, was reported. Two of these families could not be definitely categorized as to type from the data available, but in all families the homozygous individuals were indistinguishable. They excreted approximately the same highly abnormal amounts of the four amino acids and nearly all formed cystine stones. In 19 of the families the condition was recessive and no degree of abnormality was found except the extreme form in the homozygote. In 8 families the condition was incompletely recessive and the heterozygote showed moderately increased excretion of cystine and lysine without increased excretion of arginine or ornithine. Only in rare instances did the latter excrete sufficient cystine to form stones.

The simplest explanation of the two variant forms of cystinuria is that there are at least two mutant conditions of one gene. One would have a mild and the other a severe effect on the tubular reabsorption of the four amino acids in the heterozygous state. Genetic proof of this possibility must await identification of a homozygous cystinuric person, one of whose parents is the apparently normal heterozygote of the recessive type and the other a heterozygote of the incompletely recessive type of cystinuria. Families evidently of this kind are on record. Detailed chemical identification of the type of each individual in such a pedigree has not been made.



Implicit in the above figures of the relative frequency of the two types is the explanation of the rarity of cystine stone and the commonness of chemical cystinuria. The incidence of chemical cystinuria can be accepted at the maximal figure of 1 in 250 and all cases can be attributed to heterozygotes of the rare incompletely recessive form of cystinuria. Since the frequency of heterozygotes of a rare disease is approximately  $2\sqrt{\text{incidence}}$  of the homozygotes the incidence of incompletely recessive cystinuric homozygotes would be 1 in 250 000. Since recessive cystinuria has been found twice as often as the incompletely recessive type the incidence of cystinuria of both types would then be roughly 1 in 80 000. This is a reasonable figure between the maximum estimates based on crystalluria in selected cases and the ascertainment by Morner of the incidence in Sweden which set the minimal incidence at 1 in 200 000.

## DIAGNOSIS

A patient with cystine calculus may be seen at any age and at any stage of the familiar progression of renal or ureteral colic urinary tract obstruction hydronephrosis and secondary bacterial infection. Operative interferences and stone recurrences increase the risk of chronic pyelonephritis progressive renal insufficiency and finally uremia. Diagnosis depends upon chemical identification of cystine in the urine or the stone. The simple test for cystine in the urine should be performed routinely on all patients suspected of having urinary stones since the stones tend to recur and appropriate treatment may be most beneficial.

Cystine stones are about three times as dense to x rays as the poorly visualized urate stones and about equally as dense as the more common calcium magnesium and oxalate stones [9, 11]. There is nothing characteristic or diagnostic about the radiographic appearance.

### *Microscopic Crystalluria*

Microscopic examination of the urinary sediment for cystine crystals is a most useful diagnostic method. The incidence of cystine crystalluria in the population determined by this method is slightly greater than expected for the incidence of the homozygotes. Although the present tendency is to use chemical methods whenever possible it is clear that microscopic crystalluria in concentrated (morning) urines is a test sufficiently sensitive to identify the homozygotes. Since stone formation occurs only in (adult) individuals who excrete 0.4 gm or more cystine per day (2.0 mg per gm creatinine) [92] and no more than this amount dissolves in 1 liter urine at 37°C between pH 5 and 7 it follows that crystals should form when the volume of the 24 hr urine output of potential stone formers is 1 liter or less and the sample is allowed to

stand and cool for several hours before centrifugation. If crystals are sought in the relatively concentrated and acid morning urine, cystine crystals should be found in the sediment of urines from at least those cystinuric patients who might form stones. If a little glacial acetic acid is added (pH 4) and the urine refrigerated overnight, the test becomes even more reliable.

### *Chemical Tests for Cystine*

Although screening tests employing a simple determination of urinary  $\alpha$ -amino nitrogen promise to be of great value in cystinuria, the simple screening procedure for cystine used by Lewis [10] remains the basic clinical test and should be used more widely. The cyanide-nitroprusside reaction is negative with normal individuals, slightly positive with the raised amounts of cystine in urine of heterozygotes of the incompletely recessive disease, and strongly positive in urine of the homozygotes. Acetone and certain drugs can also give positive reactions.

To 5 ml urine add several drops concentrated ammonium hydroxide and 2 ml freshly prepared 5 per cent NaCN. Mix and wait 10 min for the reduction of cystine to cysteine. Add dropwise some freshly prepared 5 per cent sodium nitroprusside. A deep-purple color which fades gradually is a positive result for cystine.

The more specific Sullivan reaction can be used for confirmation. After reduction of cystine as above, add 1 ml freshly prepared 0.5 per cent 1,2-naphthaquinone-4-sodium sulfonate and mix. Add 5 ml of 20 per cent anhydrous  $\text{Na}_2\text{SO}_3$  dissolved in 0.5 N NaOH and mix. After 30 min the solution is reddish brown. One milliliter of 2 per cent  $\text{Na}_2\text{S}_2\text{O}_3$  dissolved in 0.5 N NaOH converts the color to a purer red.

The method of choice for quantitative cystine studies is polarography [51]; column chromatographic methods [81] are also good.

### *Identification of Heterozygotes and Homozygotes*

For any purposes other than the purely practical problem of clinical diagnosis, chemical identification of the specific aminoaciduria is essential. Paper chromatography will distinguish the generalized aminoacidurias of Fanconi's syndrome or Wilson's disease. Another common problem is the identification of the heterozygotes of the incompletely recessive form of cystinuria. The nature of the problem can be seen from the average values of three amino acids in the urine of normal and cystinuric individuals given in Table 42.1. The family pattern needed to indicate that the two types of cystinuria are allelomorphic should have the excretions of one parent like the controls (heterozygote-recessive type) and of one parent like the heterozygote of the incompletely recessive type and of one offspring like the homozygote. As already mentioned, quantitative cystine determinations will help in distinguishing these kinds of

individuals. The major distinction is the excretion of grossly abnormal amounts of arginine and ornithine in the homozygotes only. They also excrete high amounts of cystine and lysine. The presence of abnormal amounts of arginine and ornithine and the very high total  $\alpha$ -amino nitrogen is therefore characteristic of the homozygote. Moderate cystine and lysine excretion without these other characteristics and without generalized aminoaciduria is found in the heterozygotes of the incompletely recessive type.

## TREATMENT

Cystinuric patients enjoy excellent health and are long lived except for the complication of urinary stone formation in the homozygous individuals. The heterozygotes of the incompletely recessive form, who account for the vast majority of cystine excretors, rarely form stones and need no treatment unless their cystine excretion is over 200 mg per gm creatinine. No adverse effect is apparent from the quite considerable daily loss in the urine of the essential amino acids lysine and arginine. Subtle effects which might be seen only under stress and during unusual

TABLE 42-1 AVERAGE URINARY AMINO ACIDS

| Type and % of subjects                          | Cystine | Lysine | Arginine | As $\alpha$ -amino N |
|---|---------|--------|----------|----------------------|
| Controls (50)                                   | < 0     | < 100  | < 5      | < 10                 |
| Heterozygotes (incompletely recessive type) (6) | 1.4     | 191    | < 5      | < 28                 |
| Homozygotes (both types) (9)                    | 455     | 1055   | 39       | 181                  |

Expressed in milligrams per gram of creatinine  
Source: H. Harris et al. [10]

amino acid requirements have not been noticed. For example, the high lysine requirement for rebuilding hemoglobin after blood loss, or the high arginine supply reputedly necessary for growth of spermatozoa, might be met with difficulty by the cystinuric patient, but observations on these points have yet to be made. In one healthy 23-year-old cystinuric person, healing of a broken leg was delayed far beyond the usual time, but others have undergone major surgical procedures without difficulty. The specific aims of treatment should be to prevent initial and recurrent stone formation and, when dissolution is preferable to surgical intervention, to dissolve stones already formed. These aims appear to be attainable.

The characteristics which favor the successful treatment and prevention of cystine lithiasis also make it an ideal model of the study of the general mechanism of stone formation. Many factors of an inconstant

stand and cool for several hours before centrifugation. If crystals are sought in the relatively concentrated and acid morning urine, cystine crystals should be found in the sediment of urines from at least those cystinuric patients who might form stones. If a little glacial acetic acid is added (pH 4) and the urine refrigerated overnight, the test becomes even more reliable.

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To 3 ml urine add several drops concentrated ammonium hydroxide and 2 ml freshly prepared 5 per cent NaCN. Mix and wait 10 min for the reduction of cystine to cysteine. Add dropwise some freshly prepared 5 per cent sodium nitroprusside. A deep-purple color which fades gradually is a positive result for cystine.

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### Solubility of Cystine in Urine

Major reliance must be placed upon efforts to increase the solubility of cystine in the urine and to increase the volume of urine. It was noticed as early as 1890 that cystine is more soluble in urine than in water. Blix [97] made a careful study of the increased solubility caused by increased pH and by mineral constituents, diffusible organic materials and colloidal substances. The various salts of urine tested individually or in combination increased the amount of cystine which would dissolve by about 50 per cent but Blix attributed the major effect to colloidal substances remaining in dialyzed urine which dissolved twice as much cystine as did water.

An elegant study of the solubilizing effect of NaCl and of glycine on cystine was published by Cohn, McNeekin and Blanchard [74]. The profound solvent action of either of these substances nearly doubled the solubility of cystine but effects were not additive. Dent and Senior [16] found no increased solubility by concentrations of urea up to 10 gm per 100 ml. This was suggested by the high solubility of cystine in the urea-rich urine of the Kenya genet [69]. NaCl, other amino acids and hippuric acid also failed to increase the solubility of cystine in urine. The only prospects of producing a clinically significant increase in cystine solubility in urine appear to depend upon pH and upon possible colloidal solubilizers [70] or complexes of cystine. There is no real evidence for the existence of a more soluble complex form of cystine but in view of the striking solubilities reported in genet urine the subject warrants further study.

There is an important effect of pH on the solubility of cystine in urine (Fig. 42-9). Unfortunately little of it is in the physiologic range. From pH 5 to 7 the increase in solubility is only from 0.31 gm to 11.40 gm per liter [16]. To obtain a significant effect the urine pH must be held near the maximum of alkalinity and must certainly be above pH 7.5. This is greater alkalinity than can be produced by a high vegetable and fruit diet alone. The clinical problem is comparable to that of preventing precipitation in the kidney of the early sulfonamide compounds. Sufficient clinical experience with intensive alkali therapy is available to warrant its use at least under hospital supervision even for dissolving stones. Carbonic anhydrase inhibitors like Diamox greatly simplify the production of an alkaline urine for limited periods. Intensive alkali therapy

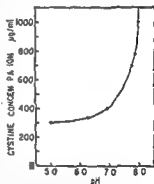


Fig. 42-9 The solubility of cystine in urine at 37°C with different pH's (Redrawn from C. E. Dent and B. Senior [16]). Cystine is about half as soluble in water.

nature, such as diet infection and urinary tract abnormalities, may be concerned with the seeding, propagation, and placement of stones in the urinary tract. At the present time however, stone formation is best considered as due to simple precipitation from supersaturated solutions. It is probable that this mechanism is the one of primary importance in cystinuria even if not in other calculous diseases, since there is a close correlation between the amount of cystine excreted and the tendency to form stones. Most individuals who excrete sufficient cystine ultimately develop stones. Certainly the concentration of cystine in the urine is the single overwhelmingly important factor in the genesis of these stones. Crystallization can be minimized by reducing the cystine concentration either by reducing the amount or by increasing the urine volume, or by increasing the solubility of cystine.

### *Quantity of Cystine in Urine*

Only a small degree of control over the amount of cystine excreted can be expected. A variety of ingenious means have been tried without success to draw cystine off into metabolic bypaths [6, 96] but the only effective way of diminishing the amount of cystine excreted is by dietary protein restriction—a method advocated for as long as cystine has been identified as a constituent of protein. The fact that more cystine is excreted on high protein diets was not fully accepted despite positive proof [25] until the experiments of Brand et al [41]. This is because the effect of protein is small and the amount of cystine in urine merely a reflection of its blood concentration. The blood level is maintained from tissue as well as dietary sources, and surpluses can be metabolized. Only the methionine in the protein not the cystine significantly raises the blood level of cystine. Severe protein restriction will only cause weight loss and negative nitrogen balance which like fever will increase the breakdown of body protein and the excretion of cystine. Excess protein should be avoided but enough must be given to replace the essential amino acids continually lost in the urine in this disease.

It is possible that high protein diets were the usual fare of two unusual patients who regularly excreted about 3 gm cystine per day at least twice the amount usually found in cystinuria [96]. Fluid and alkali therapy were inadequate to prevent stone formation in these patients and dietary measures limiting methionine intake were successfully used to decrease cystine output to the point where other therapies controlled the crystallization. The diet used was only moderately low in protein, but the proteins were substantially of vegetable origin since most of the dietary methionine is obtained from animal proteins. This diet more than halved the cystine output in these patients and was continued without harm for as long as 18 months.

normally secreted during the night constitutes the greatest hindrance to this plan

The regular occurrence of crystalluria in the concentrated morning urines of cystinuric patients was recognized early. Indeed it was believed that the formation of stones takes place only at night [7, 101, 102] and Dent and Senior made this the basis of their treatment regimen [16]. They collected separately the day and night urines of a cystinuric patient and measured the cystine excreted and the urine flow. Averaged results

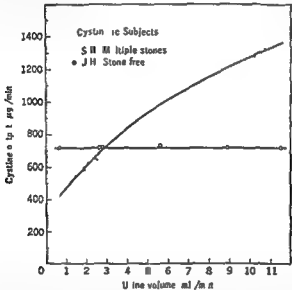


Fig 42-10 Cystine output per minute at various rates of urine flow in stone-free cystinuric (O) and cystinuric with stones (●). Increased output with higher flow in latter patient indicates dissolution of stone by diuresis. (By permission of C F Dent and B Senior [16])

from their study appear in Table 42.2. There was a constant rate of cystine excretion throughout the 24 hr. During the day the urine flow was adequate to keep the cystine in solution, but at night the decreased urine flow resulted in supersaturation and crystallization even at 37°C. Additional experiments are shown in Fig. 42.11.

Other factors may also be necessary for actual precipitation and accretion of stones. The occurrence of first stones in cystinuric persons at almost any age suggests that all the necessary conditions for stone formation are met infrequently in the day-to-day life of any one individual. It is probable that stones form and grow at night. Perhaps the dilute day urine washes away the nightly deposition. When this undependable equilibrium fails, the cystine stones can grow very rapidly indeed. Not rarely there are recurrences of cystine stones before the patient is dis-

must not be maintained too long however, because of the danger of producing phosphate stones

Alkali therapy was used as early as 1880 by Cantani [98]. He used potassium bicarbonate or citrate in his single patient. Klemperer and Jacoby used the alkali treatment successfully to dissolve a formed stone in 1914 [99]. Others have reported similar successes with sodium citrate or bicarbonate and in some instances have presented radiologic documentation of the dissolution of the stones in as short a period as 1 month. Moderate doses of sodium bicarbonate and water diuresis for 6 weeks enabled one patient to pass 284 stones weighing 10.4 gm in the aggregate [16]. The fact that alkali administration increases the solubility of cystine *in vivo* is further attested by one of the curious misconceptions perpetuated in the medical literature. When cystine excretion was recognized primarily by the presence of crystals in urine, alkali therapy was seen to dissolve the crystals and accordingly was given the credit for stopping the cystinuria. In 1929 [73] and again as late as 1935 [71] it was found necessary to prove that the disappearance of the crystals of cystine during treatment with sodium bicarbonate or citrate does not signify a reduction in cystine output. The limited surface of a stone available for solvent action gives such an unfavorable prospect that the few stones successfully dissolved away are the more remarkable. The prevention of first or recurrent stones would be better achieved by alkali and diuresis than stone removal. As discussed below, alkalinization, like diuresis, must be maintained to be effective and this requires medication during the night.

Dent and Senior were of the opinion that alkali therapy was not necessary if adequate diuresis was maintained [16]. The dissolution of cystine stones *in vivo* by diuresis alone had been reported by Weinberg and Tabenkin [100] as well as by Dent and Senior [16]. In the latter experiments the rates of cystine excretion were compared in two patients, one with renal cystine stones and one stone free (Fig. 42-10). As the rates of urine flow were increased from 1 to 11 ml per min by water diuresis, the amount of cystine excreted per minute by the stone free patient remained constant although the concentration fell considerably. The patient with cystine stones excreted more cystine per minute as the rate of urine flow increased. The concentration of cystine in the latter urine tended to be kept nearly constant by the stone cystine which dissolved.

Diuresis in the past has usually been combined with alkali therapy. The older data available do not permit crediting one or the other with successes achieved but the experiments just cited and the quantitative considerations of cystine excretion and solubility indicate that diuresis is the correct basis of therapy. Any regimen should be so designed that it can be adhered to for years without failure and that it will maintain the desired conditions in the renal collecting tubules and in the bladder throughout the 24 hr. The relatively concentrated and acidic urine



below 500  $\mu\text{g}$  per ml have been maintained in this way throughout the 24-hr period. Under these conditions there are no supersaturated urines and no stone deposition. Any stones already present slowly dissolve.

## SUMMARY

1 Cystinuria is an inherited condition of man characterized by excessive appearance in the urine of cystine, lysine, arginine, and ornithine. No other amino acids are found in excessive amounts. The condition is without deleterious effect on the subject except that cystine tends to precipitate and form stones in the urinary passages with pathologic consequences.

2 Cystine stones comprise approximately 1 per cent of all stones of the urinary passage. Cystine crystalluria is formed in approximately 0.01 per cent of pathologic urine specimens. Chemical cystinuria is found in approximately 1 out of every 250 individuals.

3 The nature of the renal tubular transport of cystine is entirely unknown. The clearance of cystine in normal subjects is approximately 3 ml per min, whereas it may approach the glomerular filtration rate in patients with cystinuria. A favored hypothesis to account for the disease is that a receptor binding substance for the four dibasic amino acids is missing in the homozygous cystinuric person and is present in reduced amounts in the heterozygote.

4 There appear to be two forms of cystinuria. In one the inheritance pattern is recessive and heterozygotes show no noteworthy excess of cystine in the urine. In the other the inheritance is incompletely recessive and heterozygotes show intermediate degrees of cystinuria and may rarely form stones. Possibly the two variants of the disease derive from variant abnormalities of allelic genes.

5 Cystinuria would be an entirely benign disease were it not for the low solubility of cystine, especially in neutral or acid solution. Preventive treatment in the homozygote is directed toward avoiding excess methionine intake, maintaining an alkaline urine by frequent use of alkali and an alkaline ash diet, and more importantly by maintaining at all times a rapid flow of urine. It is particularly important to maintain a large volume of urine during the sleeping hours when normally the urine becomes more concentrated and cystine tends to precipitate. Much can be done with this same regimen to dissolve cystine stones which have already formed.

6 Cystine excretion occurs in the Fanconi syndrome and in Wilson's disease as part of the general aminoaciduria of those diseases. It is seen in species-wide isolated aminoaciduria in the blotched genet, a wild cat of Kenya. It is seen occasionally in several varieties of the dog, but the exact nature of canine cystinuria remains to be ascertained. Recently cystine urinary stones have been encountered in the mink.

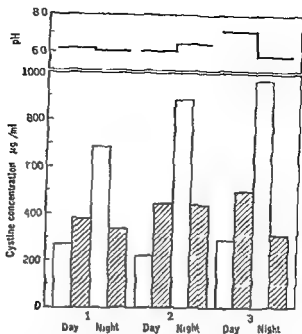


Fig 42-11 The nighttime excretion of urine super saturated with cystine pH and cystine concentrations of day and night urines of a cystinuric person over three 24-hr periods (clear columns) are shown. Amount of cystine which could dissolve in true solution in each urine is shown by hatched areas (By permission of C E Dent and B Senior [16])

charged from the hospital following a stone removal. Even high rates of diuresis of over 3 liters per 24 hr produced by daytime water dosage, do not avoid the relatively concentrated urine of the early morning hours.

TABLE 42-2 DAY AND NIGHT VARIATION IN URINARY CYSTINE CONCENTRATION IN CYSTINURIA

| Period         | Rate of excretion of |               | Cystine concentration, µg/ml |
|----------------|----------------------|---------------|------------------------------|
|                | Cystine, µg/min      | Urine, ml/min |                              |
| 6 A.M.-10 P.M. | 538                  | 2.1           | 266 (undersaturated)         |
| 10 P.M.-6 A.M. | 49                   | 0.7           | 103 (super saturated)        |

SOURCE: C E Dent and B Senior [16].

Dent and Senior recommended a regimen of diuresis which avoided the supersaturated urines of early morning. In addition to a high daytime water intake (500 ml every 4 hr) patients were instructed to drink two glasses of water at bedtime and two more at 2 A.M. Periodic tests of the cystine concentration of morning urines were used to check the success of the procedure. Urine flows of 2 ml per min with cystine concentrations

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pellagra like rash was most severe. The excretion pattern of amino acids in the urine was identical with that of her brother, E H.

It was clear then that these two siblings were affected by the same disease. An inherited condition seemed probable when it was learned that the parents were first cousins.

### *The Hartnup Family*

Neither parent and none of their six other children gave a clinical history to suggest that they were similarly affected although one girl

### *HARTNUP FAMILY - 1953*

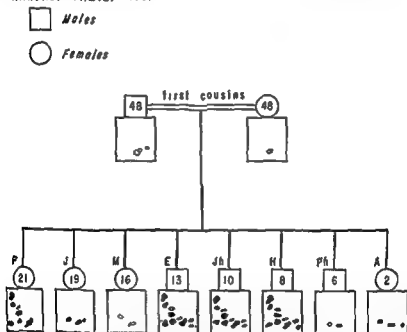


Fig 43.1 The genealogy of Hartnup disease as illustrated in the Hartnup family. The parents were first cousins. The age of the subject in 1953 is indicated by the numbers in the squares and is illustrated the chromatographic patterns of urine samples in two-dimensional chromatograms stained for amino acids.

(M H) was mentally retarded. However two younger siblings of E H, Jh H and H H, also had gross aminoaciduria with the characteristic chromatographic pattern. No abnormality was detected in the urine of the other four siblings or in either parent. In the affected children the amino acid excretion has persisted unchanged in pattern and amount up to the present. The skin and neurologic disturbances have gradually lessened in P H and E H but have made a fleeting appearance in the

## Chapter 43

### Hartnup Disease\*

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*John B Jepson and Mary Jane Spiro*

Hartnup disease is a familial condition characterized by aminoaciduria and other abnormalities apparently associated with impaired amino acid transport. The publication which first fully reported the existence of this disease [1] bore the title "Hereditary Pellagra like Skin Rash with Temporary Cerebellar Ataxia, Constant Renal Amino Aciduria and Other Bizarre Biochemical Features." As a description of the main aspects of the disease this title cannot be bettered. The family in which the condition was first found consented to the use of their surname *Hartnup*, as an appropriate appellation. The original publication [1] referred only obliquely to the name Hartnup and used the term *H disease*; occasional reference had earlier been made to *Hart's syndrome*.

#### HISTORY

In 1951 a boy aged 12 E Hartnup was admitted to the Middlesex Hospital, London Eng. with mild cerebellar ataxia and a red scaly rash on the exposed areas of his body. His mother avowed that he had pellagra for her eldest daughter (P.H.) with identical symptoms had been treated at the hospital in 1937 for that disease. Although the rash in P.H. was quite consistent with pellagra, other findings were not and a diagnosis of pellagra as a *dietary* deficiency disease was untenable.

Apart from variable cerebellar signs and retarded mental development the only abnormality detected at that time was in the urinary excretion of free amino acids. Paper chromatography of the urine disclosed an excretion pattern of amino acids quite unlike that seen in any other disease.

At about the same time P.H. now aged 19 had a recurrence of ataxia without a rash similar to that which she had had in childhood when the

\* Dr Spiro's work was supported in part by The Medical Foundation Inc.



TABLE 42.1 THE KNOWN CASES OF HARTNUP DISEASE

| Individuals | Relationship of patient to family | No. of children | Designation of patient      | Sex | Year of birth | Onset in family | Clinical (age when symptoms first noticed) |          |                        |
|-------------|-----------------------------------|-----------------|-----------------------------|-----|---------------|-----------------|--|----------|------------------------|
|             |                                   |                 |                             |     |               |                 | Itazie                                     | Flash    | Mental status          |
| [1]         | Firstborn (Hartup family)         | 8               | I H                         | F   | 1937          | 1st             | Yes (5)                                    | Yes (3)  | Retarded               |
| [1-8]       | Not reported                      | 1               | E H                         | M   | 1937          | 4th             | Yes (17)                                   | Yes (1)  | Retarded               |
|             |                                   |                 | Jb H                        | M   | 1943          | 5th             | Yes (11)                                   | Yes (8)  | Fair                   |
|             |                                   |                 | H H                         | M   | 1945          | 6th             | No   | No       | High                   |
|             |                                   |                 | Not reported (initials T S) | F   | 1949          | Only            | No   | Yes (4)  | Normal                 |
| [3-6]       | Unrelated                         | 4               | M H                         | M   | 1947          | 4th             | Slight                                     | Yes (10) | Psychotic (now normal) |
| [7-11]      | Unrelated but not specified       | 2               | Angela I                    | F   | 194           | 2d              | Yes (5)                                    | Yes (2)  | Normal                 |
|             |                                   |                 | I                           | F   | 1948          | 1th             | Yes  | Yes (3)  | Low                    |
|             |                                   |                 | Sister of I                 | F   | 1954          | 3th             | Yes  | Yes (3)  | Normal                 |
|             |                                   |                 | C                           | F   | 1947          | 1st             | Yes (5)                                    | Yes (6)  | Below normal           |
| [12-15]     | Unrelated                         | 3               | J                           | F   | 1931          | 1st             | Yes (18)                                   | Yes (3)  | High                   |
|             |                                   |                 | E                           | F   | 1937          | 2d              | Yes (12)                                   | Yes (8)  | Normal                 |
|             |                                   |                 | Candren H                   | F   | 194           | 1st             | No   | Yes (1)  | Normal                 |

*younger boys* The mental defect in M H does not seem related to the main abnormality

The pedigree of the Hartnup family (as of 1953), with diagrammatic representation of the chromatographic findings, is given in Fig 43 1 No other relatives of the Hartnup parents show the abnormality

### *Other Cases*

After publication of the definitive report on Hartnup disease in 1956 [1], two cases of 'pellagra' in English children which had been earlier reported [2, 3] were reexamined In the first case, described by Hackish [2], the dietary history was not entirely convincing The second [3 4] was a child with acute psychosis and large quantities of indican in the urine Chromatography of the urines [1] indicated that these two patients were not true dietary pellagrins but had Hartnup disease Seven other cases of Hartnup disease since discovered bring the total to 13 (Table 43 1)

## CLINICAL ASPECTS

The clinical manifestations of Hartnup disease are both intermittent and variable Although the biochemical lesion as represented by amino aciduria is always present clinically recognizable 'attacks' may occur only rarely and in widely different forms

### *Skin Lesions*

In the original Hartnup cases and in most of those found subsequently, the primary cause of referral to hospital has been a red scaly rash, sometimes dry sometimes raw and blistered The rash appears intermittently usually in summertime with a distribution on exposed parts of the face neck hands and legs suggesting photosensitivity [1 6] Hartnup patients learn to avoid exposure to direct light but photosensitivity has not been convincingly demonstrated by experiment The dermatitis closely resembles that seen in dietary pellagra in appearance and distribution

### *Neuropsychiatric Manifestations*

Several of the patients with Hartnup disease have developed a severe but fully reversible cerebellar ataxia These neurologic crises seem to be precipitated at those times when the skin rash is most severe but occasionally they may follow infectious disease without rash [1 6 7] During attacks the patient has an unsteady gait and walks with a wide base Arm movements are jerky and there is an intention tremor Nystagmus and double vision are present No sensory abnormalities are found The ataxia develops suddenly and rapidly reaches a peak The manifestation may vary from day to day Gradual improvement follows over a period of weeks and complete recovery is the rule



younger boys. The mental defect in M H does not seem related to the main abnormality.

The pedigree of the Hartnup family (as of 1953) with diagrammatic representation of the chromatographic findings is given in Fig. 43.1. No other relatives of the Hartnup parents show the abnormality.

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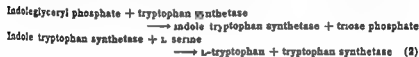
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microorganisms by utilizing various mutant strains lacking one or another of the enzymes in the sequence of tryptophan synthesizing enzymes. The synthesis of the indole ring of the tryptophan molecule appears to involve the condensation of anthranilic acid with a phosphorylated sugar derivative as shown in Eq (1)



This pathway occurs in *Escherichia coli* [9] *Aerobacter aerogenes* [10] and *Neurospora crassa* [11]. Some strains of *Saccharomyces* appear to utilize a phosphorylated hexose derivative instead of the pentose phosphate [12]. The conversion of indole and L-serine to L-tryptophan by the enzyme tryptophan synthetase, a pyridoxal phosphate requiring enzyme, has been well established [13]. Recently however Yanofsky [11] has proposed the direct utilization of the indoleglyceryl phosphate formed in Reaction (1) without prior splitting to indole and triose phosphate. The reaction as shown in Eq (2) would involve an intermediate step with the indole group attached to the enzyme.



### Degradation

The degradation of tryptophan is of particular interest because of its ultimate conversion to the physiologically important compounds nicotinic acid and serotonin. Figure 43.2 shows the many pathways by which tryptophan may be catabolized.

The overall metabolism of tryptophan in the intact animal has been assessed by the administration of  $C^{14}$  labeled tryptophan to the rat [14, 15]. Because of the rapid conversion of both uniformly labeled and ring labeled tryptophan to  $C^{14}O_2$  and to various Krebs cycle-derived amino acids, it has been proposed that the most important catabolic reaction of tryptophan may be the cleavage of the benzene ring at the level of 3-hydroxyanthranilic acid, with the formation of acetate and other aliphatic intermediates which are finally converted to  $CO_2$ . Further support for this view has been provided. Gholson and Henderson, using an acetate trapping technique [16], found the conversion of the 7  $\alpha$  carbon of tryptophan to the carboxyl carbon of acetate. In addition, Hanks and Henderson [17] found that carboxyl labeled 3-hydroxyanthranilic acid was converted to  $C^{14}O_2$  much more rapidly than could be accounted for by decarboxylation of this compound or its products.

**Formylkynurenine** Tryptophan is first converted to formylkynurenine by the enzyme tryptophan pyrrolase. This reaction has been studied in liver by Knox and Mehler [18, 19] and by Hayaishi and Stamer in

Some patients have never shown severe ataxia, but have experienced a period of "collapsing" or fainting attacks without warning [5] In the case reported by Bickel [8], cerebral involvement was limited to stubborn headache

Psychiatric features have ranged from mild emotional instability to complete delirium, a range similar to that of the psychiatric disturbances of classical pellagra They were the cause for referral to the hospital in only two cases—bizarre delusions and vivid hallucinations in one case [3], and depersonalization in the other (Patient E) [5] The intelligence quotients of the four affected children of the original Hartnup family ranged from 101 in the youngest to 61 in the eldest Baron et al [1] suggested that the biochemical lesion causes progressive mental deterioration, but cases discovered more recently do not lend support to this idea for several of the older patients have average or higher than average intelligence and ability [5, 8] Furthermore the younger Hartnup children have shown no further signs of deterioration, and the older ones have if anything improved

### *Precipitating Factors*

Attacks have been precipitated by fever [1] sulfonamide therapy [6] and psychologic stress [5] In every case there was a history of inadequate or irregular diet (e.g. one patient had lived for months largely on corn flakes [6]) It may be that clinical signs seldom appear unless under the provocation of poor nutrition A variation with age or stress in requirement for some nutrient which is in limited supply because of a biochemical abnormality might explain the clinical deterioration with growth and improvement in adult life This limiting nutrient may be nicotinic acid accordingly a discussion of tryptophan metabolism and the metabolic origins of nicotinic acid is indicated

## METABOLISM OF TRYPTOPHAN

Investigations have revealed a disturbance of tryptophan metabolism manifested by a grossly elevated excretion of urinary indican (indoxyl sulfate) and a high but variable excretion of indolylacetic acid and its conjugates in Hartnup disease In view of the relationships between tryptophan, nicotinic acid and pellagra recent investigations into the primary lesion of the disease have been concerned with the metabolism of indoles It is possible that the appearance of indoles in the urine may prove to be a manifestation of faulty amino acid transport

### *Biosynthesis*

Tryptophan is not synthesized by mammals and is therefore an essential dietary constituent Its biosynthesis has been studied in detail in

*Pseudomonas* [20] Previously the enzyme was called tryptophan peroxidase-oxidase because of its requirement for  $H_2O_2$ . Recently, Tanaka and Knox [21] have demonstrated that  $H_2O_2$  is not directly involved in the reaction but is required only in catalytic amounts for activation of the iron porphyrin portion of the enzyme. In addition Hayaishi et al [22] have found that atmospheric  $O_2$  is incorporated into the formyl kynurenine, and not  $H_2O_2$ . Considerable interest has been shown in tryptophan pyrrolase because of the ease with which increased levels of this enzyme may be induced by tryptophan administration as was originally demonstrated by Knox and Mehler [23].

**Kynurenine** The formylkynurenine produced by tryptophan pyrrolase is hydrolyzed to formic acid and kynurenine by the enzyme formylase which is also present in liver [18-19]. Kynurenine may then take part in the three pathways shown in Fig 43-2. The conversion of kynurenine to 3-hydroxykynurenine occurs in kidney and liver mitochondria in the presence of TPNH and  $O_2$  [24]. Atmospheric  $O_2$  is incorporated into the hydroxyl group in this reaction [25]. Both kynurenine and hydroxykynurenine are cleaved by the enzyme kynureninase to alanine plus anthranilic or 3-hydroxyanthranilic acid respectively. Because of the pyridoxal phosphate requirement of this enzyme [26] the metabolism of both kynurenine and hydroxykynurenine is altered in pyridoxine deficiency and appears to be shunted through the kynurenine transaminase reaction with the formation of the corresponding  $\alpha$  keto acids. These cyclize spontaneously to kynurenic and xanthurenic acids as is evidenced by the finding in the urine of pyridoxine deficient animals of large amounts of kynurenine, kynurenic acid and xanthurenic acid as well as other tryptophan derivatives in which the side chain is still intact [27].

Anthranilic acid is apparently not formed under normal circumstances and administered anthranilic acid is excreted unchanged or as various conjugates [28]. Hydroxyanthranilic acid on the other hand is already stated [17] is very active metabolically. When  $C^{14}$  labeled 3-hydroxyanthranilic acid is injected into rats the activity appears rapidly as  $CO_2$  and much smaller amounts of quinolinic acid, N-methylnicotinamide and picolinic acid. The steps by which these conversions are thought to take place involve the cleavage of the benzene ring between carbons 3 and -4 with the formation of an aldehyde intermediate which has been isolated by Wiss et al [29-30]. It is thought that enzymatic ring closure and decarboxylation occur simultaneously with the formation of either nicotinic acid or picolinic acid each by its specific enzyme. Uncertainty remains as to whether quinolinic acid is an intermediate in these reactions. Hanks and Segel who injected tritium labeled quinolinic acid into rats demonstrated the conversion of this compound to urinary N-methylnicotinamide [31]. However Mehler [32] is studying the enzyme picolinic carboxylase which converts the oxidized intermediate of hydroxy

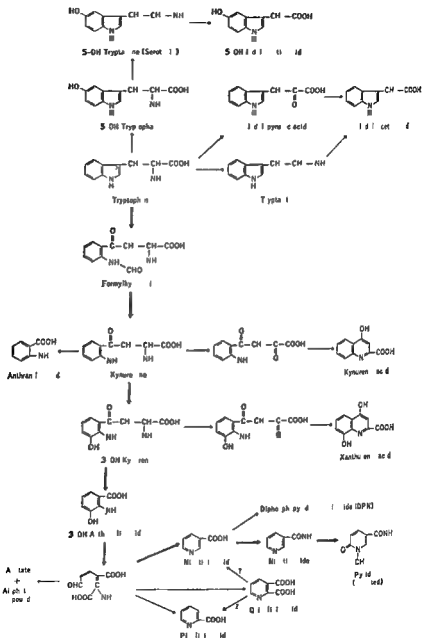


Fig. 43-2 Metabolic pathways which are open to tryptophan. Main pathways are shown in bold arrows.



## METABOLIC ABNORMALITIES

## AMINO ACIDS

*Aminoaciduria*

The pattern of the aminoaciduria in Hartnup disease is more significant than the total amount of amino acids excreted so that chromatography must be used in the diagnosis. The amounts of amino acid excreted vary from case to case and may be slightly affected by diet but are usually increased at least tenfold over the normal. This absolute amount of amino acids excreted is comparable to that sometimes reached in other types of aminoaciduria such as lanconi syndrome (see Chap 37) Table 43 2

TABLE 43-2 AMINO ACID EXCRETION IN HARTNUP DISEASE

| Patient | Weight<br>kg | Condition     | Leucine<br>mg/d                      | Alanine<br>mg/d          | Valine<br>mg/d           | Remarks            |
|---------|--------------|---------------|--------------------------------------|--------------------------|--------------------------|--------------------|
| PH      | 45           | Symptomatic   | 66 g total<br>150 mg total<br>m d/kg | 10-20 mg total<br>m d/kg | 10-20 mg total<br>m d/kg | Leucine index [46] |
| FR      | 32           | Asymptomatic  | 180 mg total<br>m d/kg               | 10-20 mg total<br>m d/kg | 10-20 mg total<br>m d/kg | [46]               |
| EH      | 32           | Dysmetabolism | 60 mg m N                            | 41 mg N                  | 41 mg N                  | Clinical trial [7] |
| ED      | 24           | Symptomatic   | 194 mg m<br>d N                      | 30 mg m<br>d N           | 30 mg m<br>d N           | Genetic [8]        |
| B       |              |               | 490 g<br>q l t                       | 34 g<br>q l t            | 34 g<br>q l t            | Clinical trial [8] |

shows some reported values. The amino acid index (percentage of total urinary nitrogen contributed by  $\alpha$  amino acid nitrogen) is five to seven times as high as normal.

The amino acid pattern as disclosed by paper and ion-exchange chromatography is quite unlike that in any other type of generalized aminoaciduria (Fig 43 3). The striking similarity of the findings in urine specimens from different patients is apparent in Fig 43 1. The amino acid pattern found in Hartnup disease is compared with that from a normal individual in Fig 43 3. Fig 43-4 shows how it appears in two different solvent systems.

The free amino acids which are excreted in amounts five to ten times normal are alanine, serine, threonine, asparagine, glutamine, valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, histidine, and citrulline. Substances which can be detected by chromatography but which are present in normal or only moderately increased amounts are taurine, glycine, cystine, aspartic acid, glutamic acid, lysine, methylhistidine, and  $\beta$  amino isobutyric acid. Even the most sensitive reagents

anthranilic acid to picolinic acid, found that quinolinic acid is not decarboxylated

**Nicotinic Acid** The enzyme system converting the oxidized intermediate of 3-hydroxyanthranilic acid to nicotinic acid has not yet been studied in detail. Mehler et al [33] have shown that the relative amounts of picolinic acid and nicotinic acid formed from hydroxyanthranilic acid depend on the relative activity of these two enzymes. In the alloxan diabetic rat the activity of the enzyme picolinic carboxylase is considerably increased. This favors a production of picolinic acid rather than nicotinic acid and accounts nicely at an enzymatic level for the decreased excretion of N-methylnicotinamide in this animal.

From a quantitative point of view very little tryptophan appears to be converted to nicotinic acid in the normal state. The dietary equivalent of 1 mg niacin is approximately 60 mg tryptophan [34].

**Serotonin** Under normal conditions the conversion of tryptophan to 5-hydroxytryptamine (serotonin) and 5-hydroxyindoleacetic acid are minor pathways. In patients with malignant carcinoid however large amounts of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid are excreted in the urine [35] and the blood level of serotonin is much increased over the normal [36]. It seems clear that serotonin is produced by the decarboxylation of 5-hydroxytryptophan rather than by hydroxylation of tryptamine [37-38]. The site of formation of 5-hydroxytryptophan is not known although the argentaffine cells have been proposed as a likely site [39]. The enzyme 5-hydroxytryptophan decarboxylase is widely distributed and requires pyridoxal phosphate [40]. Weissbach et al have demonstrated that 5-hydroxytryptamine is converted to 5-hydroxyindoleacetic acid by way of the intermediate 5-hydroxyindoleacetaldehyde [41].

**Indoles** Another pathway of tryptophan metabolism is conversion to indolic acids. Armstrong et al have shown that normal urine contains a large number of indolic acids the main ones of which are indoleacetic acid, indolelactic acid, 5-hydroxyindoleacetic acid and indoleacetyl glutamine [42]. Studies by Weissbach et al [43] have indicated that the indoleacetic acid excreted is a product of the metabolism of both intestinal microorganisms and mammalian tissues. This conversion occurs mainly by transamination of tryptophan to indolepyruvic acid with subsequent decarboxylation to indoleacetic acid. Small amounts of tryptophan are also converted to indoleacetic acid by way of tryptamine.

Another important product of the metabolism of tryptophan by the intestinal microorganisms is indole which is formed from the splitting of tryptophan to indole and pyruvic acid by the enzyme tryptophanase [44]. Indole absorbed from the intestine is hydroxylated in the 3 position to form indoxyl and is then conjugated primarily with sulfate. This detoxication occurs mainly in the liver [45].

These excretion studies indicate a highly specific disturbance of the renal tubular reabsorption of certain amino acids. No other renal abnormalities have been detected. Other clearance measurements and tests of tubular function have given perfectly normal results [1]. No reducing sugars are found. The mother of the Hartnup family showed no

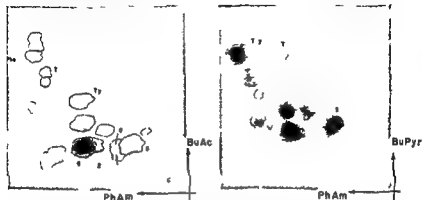


Fig 43-4 Chromatogram of a urine sample from a patient with Hartnup disease in two different solvent systems. On the left the second chromatographic system was butanol-acetic acid-water and on the right the second chromatographic system was a butanol-pyridine-water system. In each case the first chromatographic solvent system was phenol ammonium. The urine was dealted.

evidence of an abnormal amino acid clearance even in response to an oral casein load.

Although the defect in renal reabsorption is easy to demonstrate, this does not mean that it is necessarily the primary or only cause of the functional disturbances. In current opinion, based on the work of Milne [49]

TABLE 43-3 RENAL CLEARANCE OF AMINO ACIDS IN HARTNUP DISEASE

| Amino acid                   | Excretion in Hartnup disease | Clearance ml per min |        |
|------------------------------|------------------------------|----------------------|--------|
|                              |                              | Hartnup              | Normal |
| Total amino acid N           |                              |                      |        |
| Fasting                      | High                         | 6-22                 | 1.5    |
| Maximum on oral protein load | Very high                    | 25-50                | 3.0    |
| Threonine                    | High                         | 70                   | 1.0    |
| Tyrosine                     | High                         | 66                   | 1.5    |
| Histidine                    | High                         | 122                  | 6.0    |
| Taurine                      | Low                          | 1.9                  | 5.0    |
| Proline                      | Low                          | 0.3                  | 0.1    |
| Cystine                      | Low                          | 0.4                  | 0.5    |
| Lysine                       | Low                          | 3.0                  | 0.4    |

have failed to detect significant amounts of proline, hydroxyproline, methionine or arginine. The failure of Evered [46] to detect tryptophan in Hartnup urine is probably due to the destruction of this amino acid on cation exchange resins.

Perhaps the most striking differences between the Hartnup pattern and those of other generalized aminoacidurias such as Fanconi syndrome (Chap 37), Wilson's disease (Chap 25) and galactosemia (Chap 7) is

the absence of an abnormality in proline excretion. The quartet of amino acids found in cystinuria (viz, cystine, lysine, arginine, and ornithine, cf Chap 42) is excreted normally in Hartnup disease. The free amino acids are all of the L-configuration [47].

The excretion of free glutamic acid and aspartic acid is low (as in normal urine) but that of the respective amides is very high. If the urine is left at room temperature for a few hours the level of free glutamic acid rises at the expense of glutamine but specimens can be kept deep frozen for years without deterioration.

Acid hydrolysis of the urine releases no new amino acids and relatively little additional amino acid nitrogen although increased excretion of certain conjugates (e.g. hippuric acid and indolylacetylglutamine) undoubtedly occurs.

The aminoaciduria is of renal origin and not an overflow phenomenon. The renal clearance of total amino acid nitrogen both in the fasting state and following an oral load of casein is very much higher than normal [48]. Similarly the renal clearances of those individual amino acids which are excreted in excess are grossly elevated above normal although the clearances of the amino acids which are not excreted in excess are but little elevated [46]. Table 43.3 gives some examples from the work of Dent [48] and Evered [46]. The high clearance of 122 ml per min for free histidine, a figure now exceeded by the 140 ml per min in a case reported by Jonus [7] approximates the normal glomerular filtration rate and suggests a complete lack of reabsorption.

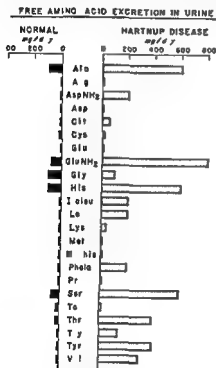


Fig 43.3 The excretion of free amino acids in the urine of normal subjects and patients with Hartnup disease.

detectable, even when L-tryptophan was fed in addition [1]. A similar result was obtained using neomycin [54] but succinyl sulfathiazole for 7 days had no effect [4, 56]. These antibiotics did not alter the urinary amino acid excretion and the indicanuria returned to its high level a few days after the cessation of treatment.

The indican could be raised considerably by oral administration of protein or L-tryptophan [1]. Milne [49] has followed this process with chromatograms of serial samples of urine. In normal subjects there was a variable rise in indican followed by a logarithmic fall but in Hartnup

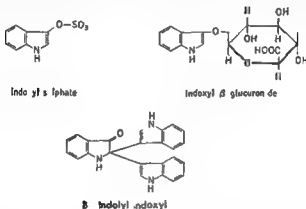


Fig 43-5 The indoxyl compounds found in the urine of patients with Hartnup disease

disease there was a greatly raised excretion which reached a maximum after 12 hr and persisted for more than 24 hr.

The urinary indoxyl derivatives presumably are final excretory products formed in the liver from indole absorbed from the gut where it arises from the action of intestinal microorganisms on unabsorbed tryptophan. The magnitude of this process in Hartnup disease could be due either to an increased growth in the gut of a particular flora or to delayed absorption of tryptophan or to both. For one might determine the other. There is little or no excretion of 5-hydroxytryptophan sulfate, the product of tryptophan formation characteristic of the malabsorption syndrome [57, 58]. Hence the responsible microorganisms may be different in Hartnup disease.

Rodnight and Mellman [4] suggested that the "pellagra" of their case was caused by the diversion of tryptophan from its normal metabolic course into the wasteful pathway of indican formation. Thus it is not the only drain of tryptophan; however, there is not only the variable loss as indican (representing 20 per cent of the daily tryptophan intake) but also the loss as free amino acid and derived indolic acids as well.

(see below) it is only one manifestation of a wider derangement of amino acid transport in this disease.

### Plasma amino acids

The amino acid concentrations, as determined by ion exchange chromatography [46] are about 30 per cent lower than normal. In the case of tryptophan, this has been confirmed by spectrophotofluorometry [50]. The following values were found for free tryptophan in plasma from three of the Hartnup boys.

| Subject | Age yr | Iminoaciduria | Plasma tryptophan $\mu\text{g/ml}$ |
|---------|--------|---------------|------------------------------------|
| Jh H    | 14     | +             | 6.2                                |
| H H     | 12     | +             | 7.8                                |
| Lh H    | 11     | -             | 10.6                               |

Westall [52] has reported that the plasma tryptophan level in normal children below 3 years of age is low, but above this age a very constant figure of  $11 \pm 1 \mu\text{g per ml}$  is found.

### METABOLITES OF TRYPTOPHAN AND INDOLE

Early in the investigations of the Hartnup family [1], a high but variable urinary excretion of indigogenic material (indican) was apparent. This led to a more complete investigation of urinary indoles by paper chromatography. A raised excretion of the indolic acids derived from tryptophan was thus revealed but this has not proved to be so constant a feature of the disease as was first thought.

### Indicanuria

All individuals with 'Hartnup' aminoaciduria also excrete large amounts of indicans. The reverse is not true. For example patients with 'blind loop syndrome' excrete much greater quantities of indicans but do not develop aminoaciduria [51]. The urinary indican from individuals with Hartnup disease is almost entirely indoxyl sulfate (I<sub>16</sub>, 41.5), but a small amount of indoxyl  $\beta$  glucuronide (I<sub>16</sub>, 43.5) can accompany it [1, 51]. Another indoxyl derivative (although not indigogenic) found in Hartnup urine is bis-indolyl indoxyl (I<sub>16</sub>, 43.5) which is invariably produced during enzymic oxidation of indole [53, 54].

The only accurate measurements of the indican excretion in Hartnup disease are those of Rodnight [1, 5]. His cases excreted 200 to 400 mg per day compared with 100 or less in normal subjects of the same age. Semiquantitative measurements based on chromatographic comparisons showed that indican excretion fell rapidly during a course of chlor tetracycline. After only 4 days on the antibiotic indican was no longer

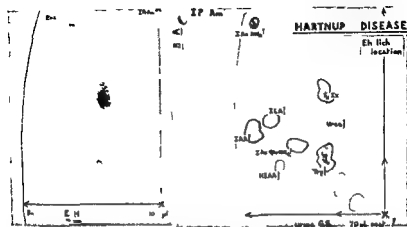


Fig 43-7 Urinary indole patterns in Hartnup disease. *Left* Paper chromatogram of urine from patient E H [2] 100  $\mu$ l urine were chromatographed with ascending solvents isopropanol ammonia first and up butanol acetic acid second right to left. Compounds were located with p-dimethylamino benzaldehyde (Ehrlich reagent). The key is found in the right hand photograph. Note the central urea spot (normally yellow) the indican spot (IS) (brown) and the triad of spots due to indolylacetic acid (IAA) indolylacetyl glutamine and tryptophan. This pattern is typical of Hartnup disease although the relative intensity of spots is variable. *Right* Paper chromatogram of urine from patient G [3] This chromatogram shows an additional spot due to indolylactic acid (ILA) not found in other cases of Hartnup disease (By permission of D N Baron et al [1] and J H Jonxis [7])

total IAA were 27 and 15 mg respectively = normal sister excreted 93 mg. The Hartnup boy (E H) who had the very high IAA excretion during 1951-1954 had a normal IAA excretion when measured in 1959 when he was 20 years old. In 1957 colorimetric determinations of free and combined IAA [49] in urines from the other Hartnup boys gave the following results [51]

| Subject | Age y | Aminoacids in | Free IAA | Conjugated IAA |
|---------|-------|---------------|----------|----------------|
| J H     | 14    | +             | 15       | ■              |
| H H     | 12    | +             | 45       | 25             |
| Ph H    | 11    | -             | 2        | ■              |

It appears that the raised IAA excretion which has been found in all cases of Hartnup disease tested before the age of 12 gradually disappears as the patient grows older although the high amino acid excretion remains.

#### Effect of Tryptophan Loading

If patients with Hartnup disease are given an oral load of tryptophan a grossly abnormal excretion of indolic acids follows [1] even if there was

### Indolylacetic Acid and Its Congeners

Paper chromatographic studies revealed that EH the first of the Hartnup family investigated, excreted indolylacetic acid (IAA) (Fig 43 6A) at a considerably higher level than normal [1] This was particularly true during his acute bouts of ataxia and rash, but even after clinical recovery the urinary abnormality was present The IAA was always accompanied by a conjugation product, originally called U1 [59] but later identified as indolylacetyl L-glutamine (IAc GluNH<sub>2</sub>) (Fig 43 6B) [60] This conjugation product is the one expected from the liver "detoxication" of IAA in man The other possible conjugate

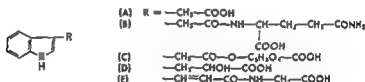


Fig 13-6. Derivatives of indole found in normal subjects and patients with Hartnup disease. See text.

indolylacetyl glucuronide (Fig. 4) [6C], has been present occasionally but only in moderate amount [8].

These indoles after two dimensional paper chromatography and location with Ehrlich reagent [69 62] showed a distribution pattern quite as characteristic as the amino acid pattern (Fig 43 7) Similar indolic patterns were found with urine from the other affected members of the Hartnup family the unaffected members had normal (barely detectable) excretion of indolic acid During one period of hospitalization the free IAA excretion in E H varied from 50 to 200 mg per day on a normal varied diet A normal excretion would be less than 10 mg per day

The constancy of the findings reported above strengthened by the discovery of similar (but variable) indolic acid excretions in some other cases [16] has been challenged by three later findings

1 In one case of Hartnup disease [8] the urinary IAA (68 mg per day) was accompanied by indolylactic acid (ILA) (Fig 43 6D) in an amount approaching that found in phenylketonuric patients (Chap 10) although no ortho- or *p*-hydroxyphenyllactic acid accompanied it [63] (Fig 43 7). ILA has not been detected in any other case of Hartnup disease.

2 The excretion of IAA as of other weak acids is influenced by the urinary pH being promoted by mild alkalosis and reduced by acidosis [49]. Measurements are not strictly comparable unless made under similar acid base conditions.

3 Two female siblings aged 23 and 24 investigated by Hersov and Rodnight [5] had no elevated excretion of IAA. Their daily excretions of



The whole blood and serum levels for serotonin (5-hydroxytryptamine) were on the low side of the normal range in parallel with the low urinary 5-HT and 5-HIA excretions [51]. This may represent a slight diversion of tryptophan from serotonin formation.

#### *Nicotinic Acid Intermediates*

Because of the pellagra like symptoms of Hartnup disease a possible enzymic block in the usual tryptophan kynurenine nicotinic acid pathway (Fig 43-2) was early suspected [1]. In one patient on a normal diet the urinary excretion of nicotinic acid and its derivatives was on the low side of normal and was surprisingly little elevated by oral administration of tryptophan [1]. The same was true of the excretion of kynurenine as seen chromatographically [51] and of kynurenic acid and xanthurenic acid in other cases [5].

Milne [49] has now compared the rise in urinary kynurenine which follows tryptophan ingestion in normal persons and in patients with Hartnup disease. Normal persons excreted 5 to 10 per cent of oral L-tryptophan as kynurenine but in Hartnup disease the conversion was only 0.5 to 1.5 per cent.

#### *Other Biochemical Investigations*

Considering the magnitude of the Hartnup amino acid lesion it is remarkable how normal the other biochemical and physiologic processes appear to remain. Two studies bear on the part played by microorganisms in the metabolic defects. In normal subjects the gut organisms are probably responsible for the production of many of the phenolic acids excreted. The urinary pattern of phenolic acids in Hartnup patients receiving a normal diet is normal [13]. Investigations of the fecal porphyrins in E. Hartnup [162] disclosed that these were generally high but that they varied in a surprising way. For example on one occasion 3 days of oral chlortetracycline lowered the protoporphyrin content of the feces from 74 to 11  $\mu\text{g}$  per gm while the same antibiotic given 1 year later raised the fecal protoporphyrin more than tenfold.

### METABOLIC LESION OF HARTNUP DISEASE

One might hope that all the clinical and biochemical features of Hartnup disease could be unified under a single metabolic block. The following biochemical findings would have to be rationalized:

- 1 The specific aminoaciduria
- 2 The retarded absorption of tryptophan from the gut
- 3 The raised production of IAA which probably diminishes with the age of the subject

normal excretion of IAA before the load [49] In normal subjects 70 mg per kg L-tryptophan causes a sharp rise in IAA and a smaller rise in ILA The peak is reached after 2 hr and the level falls to normal values in 12 hr In Hartnup disease the rise is somewhat higher and persists at a high level for at least 24 hr Residual free tryptophan may appear in the feces If DL-tryptophan is fed to normal subjects, ILA appears in the urine in large quantities It is presumably formed from absorbed L-tryptophan by the action of an amino acid oxidase

Hartnup patients react quite differently There is little excretion of ILA Most of the L-tryptophan is recovered from the feces Milne's conclusion from these experiments [49] is that there is a defective absorption of L- and of D-tryptophan from the jejunum The absorption block for D-tryptophan is thought to be virtually complete, the partial block for L-tryptophan would prolong its oxidative metabolism to IAA, but whether this occurs in the tissues or in the gut must await the results of studies after administration of antibiotics The excretion of IAA by normal subjects is only partially suppressed by neomycin or tetracycline [48] and in one Hartnup patient was actually raised by chlortetracycline [1] presumably because tryptophan was no longer diverted to indican

#### *Other Urinary Indoles*

Another indolic acid which has been found in urine of patients with Hartnup disease is indolylacryloyl glycine (Fig 43 6E) This is the expected conjugation product of indolyl acrylic acid This compound which reacts atypically with Ehrlich reagent, was first described by Kimmig [64] in urine from patients with light sensitive dermatitis It is excreted by man after the ingestion of indolylpropionic acid Urine from a Hartnup patient on a normal diet contains much more indolylacryloyl glycine than does normal urine [51] and its excretion is stimulated after an 8 hr lag period by oral administration of tryptophan [49] Chromatographic methods have failed to show indolylpyruvic acid or its decomposition products in urine [65 61, 6a]

The urinary excretion of tryptamine in Hartnup patients is normal [5] This may only be tryptamine produced locally in the kidney tubules and gives no indication of tryptamine formation in other tissues

#### *Indolic Acids in the Blood*

The whole blood and plasma indolic acids have been studied chromatographically in some cases of Hartnup disease [6 51] Apart from a slightly elevated concentration of indoxyl sulfate patients and normal subjects were indistinguishable The renal clearance values for indolic acids are normally very high [66] and there is no evidence that they are raised higher in the disease The clearance of IAA  $\text{GluNH}_2$  is at least 150 ml per min and may be synthesized at least in part from IAA in the kidney

lead to an increased metabolism along alternative pathways to indolyl pyruvic acid or tryptamine and thence to IAA. It is noteworthy that the quite different shunt of tryptophan metabolism in carcinoidosis also leads to pellagra like rashes ✓

The other biochemical abnormalities would be considered consequences of endogenous nicotinic acid deficiency at the cellular level. For example the aminoaciduria would be a result of cessation of tubular reabsorption consequent to the nicotinic acid deficiency.

It is unlikely that IAA has toxic consequences. Consumption in vast quantities as an insulinase inhibitor [72] does not cause aminoaciduria to develop [61] but IAA does have important effects on membrane permeability and amino acid uptake in plant cells.

### *Abnormal Intestinal Bacteria*

It seems improbable that the primary abnormality of Hartnup disease lies in an unusual type or concentration of microorganisms. It is much more likely that a tissue dysfunction promotes the growth of an unusual flora. Thus retention or secretion into the gut of amino acids or indoles could provide an environment favorable for abnormal flora. It is known that the oral administration of methionine causes distinct changes in the urinary indole chromatograms from Hartnup patients [60].

### *Amino Acid Transport*

The most acceptable hypothesis for the primary lesion in Hartnup disease is that the transport of amino acids into cells is defective [49]. The defect would have to involve at least the cells of the proximal renal tubules and of the jejunum in order to account for the aminoaciduria and the retention of tryptophan in the lumen of the gut. It is not yet known whether those amino acids which are poorly reabsorbed by the renal tubule are also poorly absorbed from the gut nor why some amino acids do not appear in Hartnup urine at all.

It has recently been suggested by Dickinson [73] that xanthinuria<sup>1</sup> (Chap 22) is an example of a renal defect of purine reabsorption chemically coupled with a specific xanthine oxidase deficiency, a possibility already predicted by Harris [74]. Hartnup disease may be another example i.e. the specific defect of tryptophan pyrrolase (oxidase) deficiency associated through some unknown common factor with a less specific deficiency of amino acid transport in renal and intestinal cells.

Whatever the ultimate cause of Hartnup disease may prove to be the abnormal metabolism of tryptophan may only reflect the experimental ease with which its degradation products can be measured. There is of course considerable diversion of tryptophan from its normal metabolic

4 The low experimental conversion of oral tryptophan along the nicotinic acid pathway

5 The harboring of gut microorganisms with a special aptitude for the conversion of tryptophan to indican

Three possibilities for the primary lesion are immediately obvious and were discussed in the definitive paper [1]

1 A block along the metabolic sequence from tryptophan through kynurenine to nicotinic acid

2 An intestinal habitat which supports a bacterial flora so abnormal that the metabolism of the host is affected

3 A disorder of amino acid transport in the cells of the kidney tubules and in the gut

### *Tryptophan Metabolism and Nicotinic Acid*

A considerable fraction of dietary tryptophan is normally available for the synthesis of nicotinic acid. It is possible that some tissue systems obtain their required nicotinic acid preferentially (or only) from the *in situ* metabolism of tryptophan rather than from dietary nicotinic acid. Since the conversion of dietary tryptophan to kynurenine and nicotinic acid derivatives is greatly impaired in Hartnup disease it is possible that an 'endogenous' nicotinic acid deficiency could develop. One might then account for the signs of pellagra especially under conditions of growth or stress. Nicotinamide deficiency leads to mental deterioration [68].

A genetically determined metabolic block most probably would involve tryptophan pyrrolase, an enzyme which normally catalyzes the oxidation of tryptophan to formylkynurenine [21]. This is an adaptive enzyme which is absent in fetal tissue but develops dramatically after birth. If this is the deficient enzyme in Hartnup disease the condition is a carry over of the fetal condition into postnatal life [69]. The clinical improvement in adult life might represent a gradual adaptive appearance of tryptophan pyrrolase. If the 'substrate adaptation' feature of the enzyme really is a stabilization by tryptophan [70] then the Hartnup enzyme would have an abnormal lability. On the other hand there is yet no proof of an actual deficiency of enzyme and the apparent deficiency might equally well be a reflection of reduced availability of tryptophan.

Snyder [71] has considered that Hartnup disease is due to dysfunction of an apoenzyme requiring nicotinamide as a cofactor but there is little evidence in support of this view.

The shunting of tryptophan from nicotinic acid formation would

Hartnup disease should be suspected in patients with the following signs pellagra not due to gross dietary deficiency photosensitive rash especially if accompanied by neurologic changes reversible ataxia especially when siblings are affected similarly a high excretion of indican or indolylacetic acid

## GENETICS

The 13 known cases (4 males 9 females) are distributed among 8 families with a total of 32 children (Table 43 1). Siblings are affected in 3 families 4 out of 8 in one family [1] 2 out of 3 in another [5] and 2 out of 9 in the third [7]. In 2 of these families the parents are first or second cousins 1 or 4 other families [5 6 8] no blood relationship between parents could be established even back to 1416 in the last case. Five of the families come from England 2 from Holland and 1 from Germany but no ethnic pattern is discernible. No cases have been reported in the United States.

Obviously these figures are insufficient to warrant conclusions about either mode of transmission or prevalence of the disease. If the disease is genetically determined it swells the list of those hereditary abnormalities which are not apparent at an early age but appear only under the stimulus of some external or internal metabolic influence. Although the disease appears to be rare it may be that the rarity resides in the appropriate or adequate stress rather than in the genetic existence of susceptibility.

A possibility remains that an abnormal gut flora is solely responsible for the manifestations of Hartnup disease. If this should prove to be true the condition could still be called hereditary provided that the abnormal flora were encouraged by a genetically determined vagary of the host [1].

If Hartnup disease is transmitted as an autosomal recessive trait as the original pedigree suggests affected individuals would be homozygous for the condition and both their parents would be heterozygous carriers. It might be expected that heterozygotes would show a less marked expression of the condition at the site of the primary metabolic defect. Perhaps this could be used to show which of several alternative metabolic lesions is the most likely [60].

Most of the parents and near relatives of Hartnup patients have been tested for aminoaciduria and all are uniformly negative [6]. The mother of the Hartnup children had a normal amino acid clearance even in response to a casein load [48] but a single experiment [51] suggested that she responded to an oral load of L-tryptophan with a higher than normal excretion of indolylacetic acid and a lower than normal conversion to nicotinic acid intermediates. Two of the affected girls are married but

routes and it is reasonable to suggest that the clinical aspects of Hartnup disease may result from one or more of the following

- 1 Lowered production of endogenous nicotinic acid
- 2 Increased absorption of indole from the gut
- 3 Tissue or gut formation of tryptamine [70]
- 4 Chronically low level of plasma tryptophan

## DIAGNOSIS

The only constant feature of Hartnup disease is the characteristic excretion of free amino acids and it is upon this that diagnosis must be based. The *pattern* of excreted amino acids, rather than the total amino acid excretion is the determining factor. It is not sufficient to measure total amino acid nitrogen even though a low total value would exclude the disease.

Any of the simple two dimensional paper chromatographic systems and location agents for amino acids [76] will serve. Figures 43-3 and 43-4 show the urinary amino acids expected in Hartnup disease [108]. A volume of urine corresponding to a 2 sec excretion from a 24 hr collection on a normal or low protein diet will give intensely reacting spots with the ninhydrin reagent. Normal urine shows very little. Desalting is hardly necessary with this small volume. The Hartnup amino acid pattern has never changed in any of the patients during the years they have been studied. Patients show only the most minor variation in pattern and nothing like it has been found in any other condition.

The only alternative diagnosis for Hartnup disease would be pellagra, and in the few cases of true dietary pellagra examined [1, 51] the amino acid excretion was low or normal.

The indolic excretion is not constant enough to be the basis of a diagnostic test but the abnormal response to oral L-tryptophan loading accompanied by paper chromatography of serial samples might be used [49]. Hartnup disease would be associated with a greater than normal excretion of indolylacetic acid and of indican persisting for 24 hr at least. A semiquantitative test tube method is suggestive e.g. the Obermeyer test for indican or the Weissbach test [77] for indolic acids.

Mix 1 ml urine, 2 drops concentrated HCl and 1 ml CHCl<sub>3</sub> shake well, centrifuge and reject aqueous layer, mix with 1 ml 0.5 per cent Ehrlich reagent in 12 N HCl wait 10 min, mix with 0.5 ml ethanol, add 1 drop 2.5 per cent NaNO<sub>2</sub>. An immediate blue color indicates urinary indolic acids at a level above 50 µg per ml.

Hartnup disease is not excluded by a normal indole excretion but all patients with a high indican or high IAA excretion [43] should be further examined for the Hartnup amino acid excretion pattern.

c A disorder of amino acid transport into cells particularly those of the renal tubules and the jejunum accounts for the constant aminoaciduria and for the slowed absorption of tryptophan. The latter permits an abnormally large formation in the gut of indican and may also sponsor the appearance of an unusual intestinal flora. The clinical manifestations arise through the consequent deviation of dietary tryptophan from its normal metabolic routes especially that leading to nicotinic acid.

The evidence favors the third interpretation of the abnormality of Hartnup's disease.

6 Insufficient number of patients with this disease has come to attention to permit certainty regarding its inheritance pattern but information at present is consistent with autosomal recessive transmission.

7 Patients have responded satisfactorily to oral administration of nicotinamide. A diet high in protein may be of value as well.

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neither has children. Further elucidation must await the discovery of many more cases and of a test for genetic carriers of the condition.

## TREATMENT

The paucity of cases and their variability has not made it easy to evaluate possible courses of treatment. Similarity of the symptoms to pellagra has led in many instances to oral nicotinamide therapy (40 to 200 mg amide or acid per day). Marked improvement in the dermatitis and neurologic picture has usually followed, but such improvement may occur without treatment. In view of the high urinary loss of amino acids, a high protein diet or supplement has been prescribed and seems beneficial.

## SUMMARY

1 Hartnup disease is characterized by an intermittent red scaly, pellagra like rash appearing after exposure to sunlight, attacks of cerebellar ataxia and occasionally by psychiatric changes ranging from emotional instability to delirium. Progressive mental deterioration was suggested from the original study of the Hartnup family but this has not been supported by patients subsequently encountered.

2 The disease was originally observed in 4 out of 8 offspring of a first cousin marriage. Further patients, including 3 more sets of siblings have brought the total of known cases to 13.

3 The single recognized constant feature of the disease is a massive aminoaciduria renal in origin and characteristic in pattern. This provides the only certain diagnostic test.

4 All patients excrete large but variable amounts of indoxyl sulfate (indican) in the urine. This disappears if the gut is sterilized with antibiotics. Also in most cases there are abnormally large amounts of indolyl acetic acid and its conjugates. Following oral administration of a loading amount of L-tryptophan there is a high and prolonged conversion to urinary indolylacetic acid coupled with a lower than normal conversion to kynurenine and nicotinamide.

5 Three possibilities have been considered for the primary genetically determined biochemical lesion.

a An enzymic block along the metabolic sequence from tryptophan through kynurenine to nicotinic acid with necessary diversion of tryptophan degradation. The aminoaciduria, the abnormal gut flora and the clinical symptoms develop because of a deficiency in the intrinsic production of nicotinic acid.

b An abnormal intestinal environment supports a gut flora which deranges the metabolism of the host.



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## **Part Eleven**

### **Diseases Involving a Deficiency of Circulating Enzymes or Plasma Proteins**

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## Chapter 44

### Hypophosphatasia

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*Frederic C. Bartter*

#### DEFINITION AND GENERAL DESCRIPTION

Hypophosphatasia is a familial disease characterized by abnormalities of the skeleton decreased serum alkaline phosphatase and the appearance of phosphoethanolamine in the plasma and urine. Hypercalcemia renal damage and occasionally premature synostosis of the cranial vault may also be found.

#### HISTORICAL ASPECTS

Although the disease was first given its present name by Rathbun [1] in 1948 there are scattered reports of what is undoubtedly the same entity going back 20 years before that time. The historical aspects are covered in detail in the reviews by Fraser [2] and by Currarino and associates [3]. The following reports are of especial interest in the development of currently accepted concepts of the disease. Huhne [4] in 1929 described a patient with a syndrome clearly recognizable in retrospect as hypophosphatasia. Chown [5] in 1935 described two further patients with nephrocalcinosis at autopsy. Kubatsch [6] in 1938 described a patient with islands of bone in the skull whose father had evidence of premature synostosis. Alkaline phosphatase values were not reported in any of these cases. Anspach [7] in 1939 noted hypercalcemia in a child with pathognomonic changes in the bones including premature synostosis. Serum alkaline phosphatase concentration in this child determined on a number of occasions was very low with a single exception in early infancy. Macey [8] in 1940 described two brothers (one 36 years of age) with hypophosphatasia who gave a history of severe rickets in childhood and of numerous fractures in adult life. Rathbun in his classical description of the syndrome reported that alkaline phosphatase activity was low in bone intestinal mucosa and kidney. Sobel



and increase in intracranial pressure may occur convulsions serious brain damage and death may ensue if surgical decompression is not performed The late manifestations are those of oxycephaly The sutures may show the characteristic prominent bony ridges Subperiosteal new bone formation not normally seen in rickets has been reported in three cases [1, 2 17] Premature loss of teeth is occasionally seen



A

B

Fig 44-1 X ray of the shoulder (A) and wrist (B) of a 39 month-old female with hypophosphatasia Note defects in epiphyseal ossification (By permission of Dr Edna Sobel)

Hypercalcemia is common especially in infants and hypercalciuria results The nausea and vomiting of some patients may be in part attributable to the hypercalcemia

Four cases have been observed first as adults [8 18 19] In two brothers rickets was said to have been present up to the age of 4 followed by an interim period of apparently normal health for a number of years The patients finally came to medical attention with pseudofractures one showing evidence suggestive of craniostenosis in childhood In the third adult patient rickets had been suspected in childhood The fourth had osteoporosis hypophosphatasia and phosphoethanolaminuria found

and associates [9] in 1953 considerably advanced the knowledge of this syndrome. In their patient they noted for the first time premature loss of teeth as part of the syndrome and observed that the serum did not inhibit normal phosphatase activity. They found low alkaline phosphatase activity in bone, cartilage, liver and a tooth, and finally presented data strongly suggestive of hypersensitivity to vitamin D in their patient.

Fraser and associates [10] and McCance and associates [11] discovered simultaneously in 1954, that the excretion of phosphoethanolamine in the urine is an integral feature of the disease. They showed also that the parents of patients might have abnormally low serum alkaline phosphatase values. Fraser et al [10] found that phosphoethanolamine might appear in the plasma of patients and in the urine of the otherwise normal parents of patients with hypophosphatasia. Fraser [12] also showed that the cartilage of rachitic rats would calcify in the serum of patients with hypophosphatasia but that costochondral cartilage and osteoid from a patient would not calcify in the serum of normal people or in a synthetic medium containing appropriate concentrations of calcium and phosphate ions. Scaglione [13] in 1956 reported that a patient with abnormally low phosphatase activity in bone might nevertheless have normal phosphatase activity in liver, duodenal juice, and indeed, in a tissue culture of osteoblasts. In 1958 Kretzmer and associates [14] reported alkaline phosphatase absent from the leukocytes of a patient and normal in those of the mother (whose serum phosphatase concentration was low).

#### CLINICAL DEFECTS

The clinical findings in hypophosphatasia are probably all attributable to the defect in formation of true bone to premature synostosis of the skull or to the hypercalcemia which is a frequent but not constant feature. Since the essential feature of the generalized bone disease is inadequacy of mature bone formation the clinical manifestations are more severe the earlier the appearance of the disorder. When it appears in utero infants are often stillborn and lack adequate bony support for cranial and thoracic cavities. When it appears in adult life, patients may be asymptomatic or have only symptoms attendant upon an occasional fracture. The disease of bone induces the gross changes of true rickets with beading of costochondral junctions, bowing of the legs and widening of the ends of long bones. Figure 44-1 shows the epiphysis of a child of 39 months with hypophosphatasia. The premature synostosis may be anticipated in infancy by the absence of radiologically demonstrable bone over large areas of skull giving the appearance of wide sutures separating plaques of bone. As in the syndrome of oxycephaly or acrocephalosyndactyly [15, 15a] these areas represent in fact uncalcified osteoid without fibrous septums [16]. As they calcify exophthalmos



junction of a child with the disorder [9]. The defect in appositional and subperiosteal bone formation appears as wide zones of uncalcified osteoid lined with osteoblasts. There is virtually no osteoclasts nor is there fibrosis of the marrow or other evidence of bone destruction. As in osteomalacia, the total mass of bone plus matrix may appear greater than normal [16]. The defect in membranous bone which may be so extensive as to leave only plaques of true bone in the skull [1] is histologically comparable, showing wide areas of uncalcified osteoid. It has been observed that the true bone in this disease may have a microradiologic structure relatively primitive for the patient's age and that the collagen fibrils may be correspondingly poorly arranged [20]. Material from patients with true rickets of comparable degree and severity is not available for comparison. It may be that the primitive structure results from the relative absence of remodeling of osteoid as compared with that in true bone. The first step in remodeling is, of course, bone destruction which is indicated histologically by the presence of osteoclasts. Osteoclasts are extremely rare in the bones during active rickets or osteomalacia.

### CALCIFICATION PROCESS

It is not known how the biochemical defects (decreased serum alkaline phosphatase activity, elevated plasma and urine phosphoethanolamine levels, and occasionally elevated serum and urine calcium concentration) are interrelated. The disorder may be better understood in the light of current concepts of bone physiology.

Bone formation begins with the deposition of matrix in apposition to preexisting bone or calcified cartilage or with its formation *de novo* in intramembranous sites. The osteoid is deposited by osteoblasts which contain alkaline phosphatase. Some alkaline phosphatase from osteoblasts is liberated into the circulating fluids. In the absence of liver disease, the serum alkaline phosphatase concentration closely parallels and serves as an index of the number and activity of osteoblasts.

Deposition of mineral in osteoid probably begins with the formation of an initial complex of calcium and phosphorus which in all probability is  $\text{CaHPO}_4$  [21]. The formation of this complex depends in part upon the activity product of  $\text{Ca}^{++} \times \text{HPO}_4^{--}$  surrounding the osteoid and in part upon the structure of the osteoid itself. Once formed, it is spontaneously rearranged at physiologic pH to the hydroxyapatite structure of bone. Experimental evidence regarding the essential ion product has been derived largely from studies with rachitic cartilage. It is assumed, without good evidence, that calcification of osteoid follows the same principles. Calcification does not appear in rachitic cartilage incubated in solutions with a solubility product below a critical figure. The same applies, of course, to solutions of calcium phosphate alone. The important

first in adult life with no evidence of earlier disease. It thus appears that spontaneous, virtually complete remissions may occur and that new manifestations may not appear for a number of years.

#### LABORATORY DIAGNOSIS

Laboratory diagnosis consists of (1) demonstration of serum alkaline phosphatase values below normal for the patient's age, (2) histologic characterization of the bone lesion, (3) demonstration of phosphoethanolamine in the urine. This compound has not been identified in



Fig. 44-2 Costochondral junction of a rib of a 33-month-old girl with hypophosphatemia. Note disorganization of cartilage and osteoid seams lined with osteoblasts. Undecalcified. (By permission of Dr. Edna Sobel.)

the urine of normal subjects. Quantitative data for phosphoethanolamine in urine or plasma in hypophosphatemia are not yet available.

Chemical analysis may also allow the recognition of hypophosphatemia in persons with no signs or symptoms of the disease. Relatives of patients may have low serum alkaline phosphatase values and phosphoethanolamine in the urine (see below). These persons presumably are heterozygous for hypophosphatemia.

The histologic picture is generally indistinguishable from that of true rickets. The defect in ossification from cartilage appears as in rickets, as a widening of the zones of provisional calcification, disruption of the normal columnar arrangement of cells, and failure of calcification of degenerating cartilage. Figure 44-2 shows a section from a costochondral

phatase in the calcification process however since it has been shown that beryllium has this property in concentrations which do not affect liberation of inorganic phosphate from  $\beta$  glycerophosphate [27]

Finally it has been suggested that phosphatase serves to destroy phosphate esters which inhibit calcification as crystal poisons [28] In this view, the phosphate esters which are promoting calcification in slice experiments 'protect' phosphate-containing sites from phosphatase leached from the slices [21]

## BIOCHEMICAL DEFECTS IN HYPOPHOSPHATASIA

Although rachitic cartilage (and probably the uncalcified osteoid in osteomalacia) normally calcifies readily in normal concentrations of calcium and phosphate ions this does not occur in patients with hypophosphatasia. In this syndrome the serum phosphorus level is normal and the serum calcium level is frequently elevated despite the presence of histologic rickets. Furthermore Fraser showed directly that rachitic cartilage would calcify in the serum of a patient with hypophosphatasia. This makes it unlikely that the defect in calcification results from too low an activity product of circulating calcium and phosphate ions. The possibility does exist however that calcium may be present in a complexed nonionized form. This is suggested by the observation that vitamin D in average doses may rapidly elevate the serum calcium concentration of patients with hypophosphatasia [9]. Anning and associates [9] showed that vitamin D can increase significantly the concentration of diffusible nonionized calcium. On the other hand if calcification depends upon the activity of phosphate locally at osteoid sites and this is dependent upon phosphatase the disorder may indeed involve a decrease of effective ion product.

The syndrome thus provides strong clinical evidence that alkaline phosphatase plays a role in the calcification process. Since osteoid is present in abundance it appears likely that phosphatase is not required for its formation. There remains the possibility that osteoid formed without alkaline phosphatase is abnormal e.g. lacks a phosphorus-containing compound required for *in vivo* calcification such as the mucopolysaccharide described by DiStefano and associates [30]. As noted above the rachitic cartilage of a patient with hypophosphatasia did not calcify in normal serum [12]. Attempts to define further the relationship between the enzyme deficiency and the bone disease have proved unsuccessful.

An inhibitor of alkaline phosphatase could not be found in the serum of patients although carefully sought for by four groups of investigators [9 10 16 20]

point is that calcification *in vivo* normally occurs despite a product of activities of  $\text{Ca}^{++} \times \text{HPO}_4^{--}$  in serum below that required for spontaneous precipitation (Hydroxyapatite the mineral phase of formed bone will remove calcium and phosphate from solutions with activity product  $\text{Ca}^{++} \times \text{HPO}_4^{--}$  below that of serum. The present problem however, concerns a failure of calcification *de novo* of newly formed osteoid or "calcifiable" cartilage.)

A number of attempts have been made to explain this paradox. The first attempt (which antedated indeed accurate knowledge of the appropriate activity products) was that of Robison [22] who noted the presence of alkaline phosphatase in all areas where calcification occurs. He suggested that phosphatase acts to liberate inorganic phosphate at the site of calcification and thus by raising the activity product of calcium by phosphate ions locally, to initiate crystallization. This view was strengthened by the observation that calcification of rachitic cartilage would not occur after inactivation of phosphatase, save at concentrations of calcium and phosphate ions far above the physiologic range [23]. The concept was rendered untenable, however by the demonstration that inhibitors of glycolysis not affecting alkaline phosphatase could inhibit calcification of cartilage in concentrations of calcium and phosphate well above the physiologic range. It was shown that calcification is blocked by phlorizin, iodoacetate or fluoride but that it can be restored in the presence of the first two if glucose 1 phosphate or 3 phosphoglycerate respectively, is added. These compounds appear "below" the block in glycolysis. ATP itself would not promote calcification so it does not appear that the glycolytic cycle serves solely to supply energy [24].

Further this inhibition can be overcome by the addition of substrates (such as phenylphosphate) not in the pathway of glycolysis but readily hydrolyzed by phosphatase. These observations led Gutman and Yu to propose that phosphatase acts as a transferase of phosphate from organic phosphate esters to available sites on the matrix [24].

Two other theories have been proposed to explain the place of phosphatase in bone formation. It has been suggested that it has no direct role in calcification but is associated with formation of organic matrix. This view finds strong support in the recent demonstration that calcification of cartilage reconstituted from solution will occur spontaneously at physiologic concentrations of calcium and phosphorus [25]. The only requirement is that the cartilage (whatever the source) have the structure of 'native' cartilage. This poses the question as to why all native cartilage does not calcify. It has been suggested that traces of an essential mucopolysaccharide [26] are present even in these purified solutions. It has been shown that beryllium which inhibits alkaline phosphatase activity inhibits calcification. This does not establish a role for phos-

phatase in the calcification process however since it has been shown that beryllium has this property in concentrations which do not affect liberation of inorganic phosphate from  $\beta$  glycerophosphate [27]

Finally it has been suggested that phosphatase serves to destroy phosphate esters which inhibit calcification as crystal poisons [28] In this view the phosphate esters which are promoting calcification in slice experiments protect phosphate-containing sites from phosphatase leached from the slices [21]

## BIOCHEMICAL DEFECTS IN HYPOPHOSPHATASIA

Although rachitic cartilage (and probably the uncalcified osteoid in osteomalacia) normally calcifies readily in normal concentrations of calcium and phosphate ions this does not occur in patients with hypophosphatasia In this syndrome the serum phosphorus level is normal and the serum calcium level is frequently elevated despite the presence of histologic rickets Furthermore Fraser showed directly that rachitic cartilage would calcify in the serum of a patient with hypophosphatasia This makes it unlikely that the defect in calcification results from too low an activity product of circulating calcium and phosphate ions The possibility does exist however that calcium may be present in a complexed nonionized form This is suggested by the observation that vitamin D in average doses may rapidly elevate the serum calcium concentration of patients with hypophosphatasia [9] Arning and associates [29] showed that vitamin D can increase significantly the concentration of diffusible nonionized calcium On the other hand if calcification depends upon the activity of phosphate locally at osteoid sites and this is dependent upon phosphatase the disorder may indeed involve a decrease of effective ion product

The syndrome thus provides strong clinical evidence that alkaline phosphatase plays a role in the calcification process Since osteoid is present in abundance it appears likely that phosphatase is not required for its formation There remains the possibility that osteoid formed without alkaline phosphatase is abnormal e.g. lacks a phosphorus-containing compound required for *in vivo* calcification such as the mucopolysaccharide described by Dr Stefano and associates [30] As noted above the rachitic cartilage of a patient with hypophosphatasia did not calcify in normal serum [12] Attempts to define further the relationship between the enzyme deficiency and the bone disease have proved unsuccessful

An inhibitor of alkaline phosphatase could not be found in the serum of patients although carefully sought for by four groups of investigators [9 10 16 20]

point is that calcification *in vivo* normally occurs despite a product of activities of  $\text{Ca}^{++} \times \text{HPO}_4^{--}$  in serum below that required for spontaneous precipitation (Hydroxyapatite the mineral phase of formed bone will remove calcium and phosphate from solutions with activity product  $\text{Ca}^{++} \times \text{HPO}_4^{--}$  below that of serum. The present problem however, concerns a failure of calcification *de novo* of newly formed osteoid or calcifiable cartilage.)

A number of attempts have been made to explain this paradox. The first attempt (which antedated indeed, accurate knowledge of the appropriate activity products) was that of Robison [22] who noted the presence of alkaline phosphatase in all areas where calcification occurs. He suggested that phosphatase acts to liberate inorganic phosphate at the site of calcification and thus, by raising the activity product of calcium by phosphate ions locally to initiate crystallization. This view was strengthened by the observation that calcification of rachitic cartilage would not occur after inactivation of phosphatase save at concentrations of calcium and phosphate ions far above the physiologic range [23]. The concept was rendered untenable, however by the demonstration that inhibitors of glycolysis not affecting alkaline phosphatase could inhibit calcification of cartilage in concentrations of calcium and phosphate well above the physiologic range. It was shown that calcification is blocked by phlorizin, iodoacetate or fluoride but that it can be restored in the presence of the first two if glucose 1 phosphate or 3 phosphoglycerate respectively is added. These compounds appear "below" the block in glycolysis. ATP itself would not promote calcification so it does not appear that the glycolytic cycle serves solely to supply energy [24].

Further, this inhibition can be overcome by the addition of substrates (such as phenylphosphate) not in the pathway of glycolysis but readily hydrolyzed by phosphatase. These observations led Gutman and Yu to propose that phosphatase acts as a transferase of phosphate from organic phosphate esters to available sites on the matrix [24].

Two other theories have been proposed to explain the place of phosphatase in bone formation. It has been suggested that it has no direct role in calcification but is associated with formation of organic matrix. This view finds strong support in the recent demonstration that calcification of cartilage reconstituted from solution will occur spontaneously at physiologic concentrations of calcium and phosphorus [25]. The only requirement is that the cartilage (whatever the source) have the structure of native cartilage. This poses the question as to why all native cartilage does not calcify. It has been suggested that traces of an essential mucopolysaccharide [26] are present even in these purified solutions. It has been shown that beryllium which inhibits alkaline phosphatase activity inhibits calcification. This does not establish a role for phos

### THE RAPY

There is no generally accepted therapy for hypophosphatemia. Vitamin D has been tried (because the bone disease is the same as that found in rickets) with apparent improvement in one case [17]. This same case and several other cases so treated [2, 9, 36] developed hypercalcemia and signs of vitamin D intoxication. There is no further evidence that vitamin D deficiency plays a part in the disease or that therapy with vitamin D is effective.

In a single case, cortisone has been reported to produce rapid improvement in the radiologic defects in the bones and to raise the serum alkaline phosphatase concentration to twice pretreatment values, although not to normal levels. Relapse occurred when cortisone was omitted and there was a comparable response when it was reintituted. On the other hand, four cases are said to have shown no response to cortisone [18, 37-39].

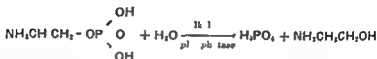
There is no record of the use of phosphate supplements as a therapeutic measure to induce hyperphosphatemia. As noted above, it is possible that acalification in hypophosphatemia results from a subnormal ion product at osteoid sites normally overcome through an action of phosphatase. It is possible that the defect could be overcome by inducing an abnormally high solubility product in circulating fluids.

### GENETIC ASPECTS

Figure 43-3 shows the family tree given by McCance and associates [16] who studied two siblings who died in infancy. It is of interest that low serum alkaline phosphatase values were found in two generations. There was strong circumstantial evidence for the occurrence of hypophosphatemia in the maternal sibship of the father of the propositi.

An analysis of the genetic aspects of hypophosphatemia by Steinberg is quoted in a recent review [3]. He estimates that the incidence of the disease among siblings of probands is 23 per cent, notes no evidence of sex linkage, and finds significantly more affected females than males. From data based on serum phosphatase values alone, he makes the following analysis. The serum phosphatase level is low in 70 per cent of affected parents for whom data are available. Assuming that the disease is transmitted as an autosomal recessive trait (and that all parents of patients are thus heterozygotic for hypophosphatemia), two-thirds of all clinically normal siblings of patients should be heterozygotic and about 50 per cent ( $0.70 \times 0.66$ ) should then have low serum phosphatase values. This compares well with an observed ratio of three affected in seven clinically healthy siblings tested. Steinberg further noted a high frequency of the disease in the last born sibling and concludes that it is

Phosphoethanolamine has been isolated from urine and plasma of patients and of some parents of patients. Although alkaline phosphatase readily hydrolyzes this ester, there is no reasonable explanation for the association of the two biochemical defects in the disease. It has been suggested that phosphoethanolamine is the true substrate for bone alkaline phosphatase [19]. Thus, a hereditary block in the reaction



would lead to an excess of plasma and urinary phosphoethanolamine. A block in hydrolysis of the ester could lead to a defect in calcification whether the phosphatase acts as a liberator of inorganic phosphate as a transferase or indeed, as a metabolic "poison" as suggested by Neuman [28]. There is no evidence in favor of or against such a hypothesis. Phosphoethanolamine is not normally found in serum or urine. It is a normal constituent of brain tissue. It may appear with amino acids in the urine of patients with liver disease [31], celiac disease and erythroblastosis fetalis [32].

Hypercalcemia and hypercalciuria occur frequently but not constantly in hypophosphatasia. When they do occur there may be nitrogen retention and nephrocalcinosis. A serum calcium concentration greater than 11 mg per 100 ml was noted on one or more occasions in 20 of the 31 cases for whom data were available [2].

It has been observed [1] that the serum calcium may undergo wide fluctuations without apparent cause and [2] that hypercalcemia may result from therapy with moderate doses of vitamin D. It may also occur in subjects who have never received vitamin D [3]. Thus one patient developed a serum calcium concentration of 17 mg per 100 ml after 10 days on 50 000 units a day [9], another who had a serum calcium concentration of 12 to 15 mg per 100 ml before therapy with the vitamin showed a return of serum calcium level to normal while the patient was receiving 50 000 units a day [17].

Inasmuch as the bones are surrounded with osteoid and little or no osteoclastic activity is seen histologically it has been generally assumed that the hypercalcemia results from hyperabsorption of calcium. Even with normal calcium absorption hypercalcemia might indeed develop if the bone matrix were unable to deposit calcium (as is apparently the case in hypophosphatasia). The occurrence of all the other abnormalities of hypophosphatasia in many patients with normal serum calcium who have not had vitamin D throughout their clinical course eliminates the possibility that the alkaline phosphatase in this disease is depressed as a result of hypervitaminosis D and hypercalcemia [33-34].



activity is decreased and phosphoethanolamine is found in plasma and urine. There may be premature synostosis of the cranial vault, hypercalcaemia, nephrocalcinosis and premature loss of teeth.

2 The disease may be detected first in infancy or in adulthood. Diagnosis is based on the demonstration of radiologic and histologic changes in bone indistinguishable from those of rickets, phosphoethanolamine in the urine and decreased serum alkaline phosphatase activity.

3 The cardinal pathologic feature is inadequate calcification of bone matrix. The biochemical basis for this and its relation to the other biochemical defects are not known. It is possible that alkaline phosphatase is necessary for the formation of normal osteoid or that phosphoethanolamine normally has an integral place in the calcification process.

4 The disease picture offers strong support for a role for alkaline phosphatase in normal calcification of bone.

5 There is no adequate therapy.

6 Available data suggest that the disease is inherited as an autosomal recessive trait. Heterozygotes may have low serum alkaline phosphatase concentration, high urinary excretion of phosphoethanolamine or both without bone disease.

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possible that heredity may not be involved in the disease. It may be of course that families with an affected child avoid future pregnancies or that medical reports appear before the families are complete.

The analysis on which Steinberg's study was based does not include more recent data on phosphoethanolamine excretion among ancestors and siblings of patients. When this valuable adjunct to the detection of heterozygosity is incorporated into a larger genetic study the hereditary

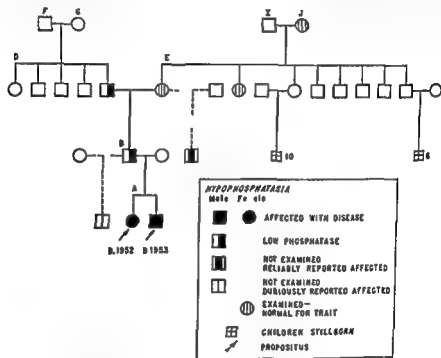


Fig 44-3 Pedigree of two infants with hypophosphatasia (By permission of R A McCance et al [16])

aspects of hypophosphatasia should be considerably clarified [2]. Fraser reported that this test brought the incidence of detectable heterozygosity in parents of patients to 81 per cent. He notes that there is no good evidence that the defect of phosphatase and that of phosphoethanolamine metabolism are carried on the same gene and that there is suggestive evidence that they are not. Relatives who excrete phosphoethanolamine tend to have higher serum phosphatase values than those who do not.

## SUMMARY

1 Hypophosphatasia is a familial disease in which severe skeletal defects result from a failure of deposition of apatite in the end and of normal ossification at epiphyseal plates. The serum alkaline phosphatase

## Chapter 45

### Hereditary Hypoproteinemias and Other Plasma Protein Abnormalities

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*Robert S. Gordon, Jr.*

During the past decade the availability of isotopic tracers and the application of the concept of the dynamic state of body constituents [1] together with increasingly effective procedures for the determination and isolation of specific proteins [2] have led to significant advances in the understanding of the physiology and biochemistry of the plasma proteins. At the same time improved clinical chemical methods for the estimation of proteins and the great simplification of electrophoresis by the introduction of supporting media have stimulated the application of this new knowledge to the study of disease [3]. It is not surprising therefore that this decade has been the period of discovery of disorders characterized by abnormal metabolism of plasma proteins. Some of the important defects in plasma protein metabolism will be discussed in this chapter. In these conditions deficiency or qualitative abnormality of the plasma protein is demonstrable by physical methods.

Before the disorders of plasma protein metabolism are introduced it will be desirable to outline the normal metabolism of these constituents. No plasma protein has been so extensively studied with regard to origin, turnover and metabolic fate as albumin. Its natural abundance, relatively high stability, the fact that it may be purified by crystallization and the availability of large quantities for intravenous administration to man make it particularly suitable for metabolic investigations. For these reasons its metabolism will be discussed as an example of plasma protein turnover. Far less is known of the turnover of the other plasma proteins although it seems reasonable to assume that their metabolism is similar to that of albumin with different rates of formation and destruction or utilization. Gamma globulin has been shown to differ from albumin in one important respect: it originates in peripheral tissues [4] probably in

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with an amino acid sequence that appears to deviate from the normal only with regard to a small number of residue but in which the deviant synthesis is itself specific and is passed on unchanged from generation to generation.

### *Distribution and Catabolism of Albumin*

The distribution of serum albumin in man has been shown [16-17] to involve a volume considerably greater than that of the circulating plasma. The latter contains only about 40 per cent of the total exchangeable albumin. The presence of plasma proteins in lymph was emphasized many years ago by Drinker and Lield [18] and the existence of immunologically identifiable plasma proteins in the interstitial fluid of muscle has been shown by Gitlin, Landing, and Whipple [19]. Albumin degradation appears to be a first-order process and a newly synthesized molecule is just as likely to be degraded as an old one. A semilogarithmic plot of the disappearance of labeled albumin yields a straight line, and the survival of the protein is measured by a half time.

A recent study of the degradation of  $I^{125}$  labeled albumin in man [20] indicates that the site of the metabolism of albumin is an extravascular compartment which is in rapid equilibrium with the circulating albumin. Animal experiments [21] suggest that the liver plays an important role in the catabolism as well as in the synthesis of albumin *in vivo* and studies *in vitro* with surviving perfused liver [22] and with slices [23] demonstrate hepatic albumin degradation. The further fate of compounds released by the degradation of albumin *in vivo* is not known although it has been shown in the intact animal that labeled amino acids from albumin eventually make their way into new proteins [24-25]. Albright et al. [26] and Waterhouse, Bassett, and Holler [27] suggest that albumin contributes to the formation of new protoplasm in human subjects. In balance studies on subjects receiving albumin by vein they find retention of phosphorus which so far exceeds the retention of calcium that it cannot all be accounted for by bone formation.

### *Clinical Investigation of Albumin Metabolism*

Luck [28] has described in sobering detail the problems confronting the clinical investigator of serum protein turnover. An isotopically labeled protein is introduced into the circulation of the subject to be investigated and its rate of disappearance is followed. The performance of such a study is complicated from the start by the difficulties inherent in the isolation of a pure protein for administration and for isotopic assay. Other difficulties are inherent in the biologic behavior of the labeled protein. If it is tagged with  $N^{15}$ ,  $C^{14}$  or  $S^{35}$  the commonly used isotopes of the elements

plasma cells [6]. In addition, most of the plasma proteins cannot be isolated as single components for investigation. The heterogeneity of  $\gamma$  globulin as usually defined seems to be reflected in a range of half times in survival studies [6].

## METABOLISM OF SERUM ALBUMIN

### *Chemistry of Albumin*

Serum albumin in crystalline form has been available for chemical investigation since the work of Cohn, Hughes, and Weare [7] in 1947. The studies that have been performed on this protein have made it by far the best known of the plasma proteins, although its detailed structure has not yet been defined. It probably consists of a single peptide chain, internally cross-linked by disulfide bonds between cysteine residues [8]. The terminal amino acids have been tentatively identified for human serum albumin: the N-terminal residue is aspartic acid [9, 10], followed by alanine. The C-terminal acid is leucine, with (albumin) glycine valine alanine leucine being the probable sequence of preceding residues [11]. A discussion of the many physical characteristics and chemical properties of serum albumin that have been reported is beyond the scope of this chapter and is not essential for the present consideration of disturbances in its metabolism. Edsall and Wyman [12] have incorporated the significant features of the physical chemistry of serum albumin into their recent monograph.

### *Synthesis of Albumin*

Beyond the observation that incorporation of  $C^{14}$  lysine into serum albumin occurs exclusively in the liver of the rat [13], virtually nothing is known of the details of its synthesis. The processes of protein synthesis and their genetic control have been intensively investigated in systems simpler than mammalian liver; this field has been reviewed recently by Anfinsen [14].

The question of the specificity of protein synthesis and structure is of the greatest importance in a discussion of the disorders of serum protein metabolism. In a current review of this field, Vaughan and Steinberg [15] advance the belief that all the proteins that have been adequately characterized are synthesized by mechanisms of high specificity, so that each molecule of the protein under consideration is identical with each other, at least as regards amino acid sequence. This tenet is supported by the apparent homogeneity throughout a wide variety of studies of human and bovine serum albumins, even when the proteins are prepared from pooled serums representing many individuals. This concept of specificity of chemical structure of the protein renders intelligible the investigations of the hereditary variation discussed below, in which an albumin occurs

## ABNORMALITIES OF SERUM ALBUMIN METABOLISM

## DOUBLE ALBUMIN ANOMALY (BISALBUMINEMIA)

This condition which cannot properly be called a disease has been described in at least six kindreds within the past 25 years. Since the abnormality can be detected only by electrophoretic analysis of the plasma proteins and since it appears to produce no untoward effects in its bearers, it is hardly surprising that it had not come to medical attention earlier. As an inherited trait which can be measured quantitatively, it is of interest to the geneticist, and familiarity with the condition may prevent unnecessary excitement on the part of physicians who encounter the anomaly in the future. It seems appropriate to mention it in connection with other disorders of plasma protein metabolism.

The first mention of the anomaly was made by Scheurlen [30] who noticed a double albumin peak in the electrophoretic diagram of the plasma proteins of one of a series of diabetic patients. This case may actually not have been typical since the separation of the two peaks in the electrophoretic diagram appears to have varied with changes in the clinical condition of the patient. In a later report [36] it is stated that the same anomaly was encountered in two relatives of this original patient and the anomaly is equated by the authors with more clear-cut examples of the disorder. Subsequent reports by Knedel [37-38], Nennstiel and Becht [39], Earle, Hutt, Schmid and Gitlin [40-41] and by Bennhold, Ott and Scheurlen [36] have brought to six the number of kindreds in which the condition is recognized. The family studied by Earle et al. is the largest, containing 20 proved and 3 presumed cases. These authors have carried out studies of the pattern of heredity and of the chemistry of the abnormal protein. The discussion which follows is based primarily on their observations.

*Biochemical Features of Albumin II*

The anomaly consists of a double albumin peak in the electrophoretic pattern of serum. The combined areas are equal to the area of a normal albumin component. The faster moving component has the mobility of normal albumin and cannot be differentiated from it; the slower moving component (called albumin B by Earle et al.) is superimposed on the  $\alpha_1$  globulin. The two albumins do not separate in the ultracentrifuge, are both found in Cohn fraction V, and become equal in electrophoretic mobility at pH values above 11.3 and below 3.5. The quantitative precipitation reaction with rabbit antiserum and the Ouchterlony agar diffusion technique do not differentiate albumin II from normal human serum albumin. These observations may be explained satisfactorily by the hypothesis that albumin B is in fact identical with normal serum albumin.

naturally occurring in proteins the investigator must estimate the extent to which these isotopes liberated by the breakdown of body or plasma protein may be reutilized in the synthesis of new protein. If, on the other hand, it bears  $I^{131}$  the most commonly employed extraneous isotopic label there is the ever present possibility that the labeled molecules may be differentiated metabolically from the natural ones. These considerations make it impossible to obtain an absolutely precise measurement of the rate of albumin turnover in man but they detract only slightly from the usefulness of labeled proteins in the study of clinical problems associated with disturbed albumin metabolism. By applying the same procedure to subjects under study and to normal controls one can at least make a valid estimate of the direction and extent of the differences encountered.

In the procedure of Armstrong et al [29-30], the patients label their own proteins after ingestion of isotopic precursors contained in yeast grown in the presence of  $S^{34}$ . The rates of incorporation and loss of isotope from the circulating plasma proteins can then be followed. It is apparent that this technique does not permit the calculation of total body albumin content by isotope dilution. A further disadvantage stems from the labeling of all body proteins and in fact all sulfur compounds undergoing active metabolism so that there is a very considerable pool of isotope available for reincorporation into plasma proteins as it is released. For this reason, the absolute rates of catabolism of albumin estimated by this procedure are erroneously low, but the procedure is a simple one, and probably suitable for the comparison of patients with control subjects. Many of the objections that might be raised to the above mentioned procedure were avoided by Volwiler et al [31], who administered isotopic methionine to one subject and then transferred labeled plasma proteins from this subject to others whose turnover rate was to be investigated. Unfortunately this procedure is too cumbersome for widespread clinical use and involves hazards (of radiation for the plasma donor and hepatitis for the recipient) that sharply limit its applicability.

In contrast to the techniques just described which use isotopes of atoms naturally occurring in proteins the most popular procedure [16-17-20] has been the study of albumin distribution and degradation using protein labeled with  $I^{131}$ . Cohen et al [32] and Campbell et al [33] have presented evidence that  $I^{131}$  labeled protein behaves very much like  $C^{14}$  protein in experimental animals and a recent study by Freeman et al [34] demonstrates that  $I^{131}$  albumin and native albumin are degraded at the same rate in a patient with analbuminemia. The easy availability of  $I^{131}$  albumin, the simplicity and accuracy of its assay and the advantages of the isotope in minimizing radiation hazard to the patient have made this compound a favorite tool for clinical research.



kindreds 13 of 18 have the anomaly. When one remembers that there are necessarily 6 affected propositi it may be seen that the probability of inheriting the trait from an affected parent is not significantly different from 0.5. The data are interpreted by Finkle et al. as consistent with the hypothesis that there are two genetic loci controlling the synthesis of albumin that these loci are independent and that in the heterozygote there is equal production of normal albumin and albumin B. These authors consider that the anomaly is transmitted as a codominant characteristic both the abnormal and the normal genes being fully expressed. The homozygotic abnormal situation has not been encountered to date. Whether or not such a homozygotic individual would suffer from some clinically detectable disease cannot be predicted on the basis of presently available information.

### ANALBUMINEMIA

#### *Historical Aspects*

Bennhold Peters and Roth [42] first described a pair of siblings from whose plasma albumin was absent. Subsequent communications from the clinic at Tübingen [43-44] have amplified the original description and presented the results of a variety of biochemical investigations. Recently three more analbuminemic subjects have been reported: two in the United States and one in Switzerland [45-47]. The former two patients have been under observation since 1952 and 1956 respectively; the latter was only recently discovered. Pending the identification of more individuals with this disorder the description of the disease must be based on this small series and generalizations must be regarded as tentative.

#### *Clinical Features*

The most striking feature of analbuminemia is the relative paucity of symptoms and signs. Edema, usually of mild degree, has been noted in all cases but one and has been relieved by the administration of albumin in the two individuals who received full replacement doses. The significance of edema cannot be assessed in the patient reported by Shetlar et al. [47] because of the coincident occurrence of congestive heart failure. Two subjects [44, 45] have received replacement therapy with large amounts of exogenous human serum albumin and have noted an increased sense of well-being which may be taken as evidence of a preexisting mild malaise. The two young male patients, however, feel well enough to carry on heavy manual labor [43, 46]. Bennhold and his colleagues [42] observed that their two patients with analbuminemia had a moderate degree of arterial hypotension, a feature which they considered to be an adaptation which helps to prevent excessive edema for

except that two or more cysteine or tyrosine residues replace residues of dicarboxylic acids in the structure of the molecule

Individuals who have the albumin II anomaly quite uniformly have approximately equal quantities of normal albumin and of albumin B. Earle et al state that albumin II constitutes  $57 \pm 4.4$  per cent of the total albumin. The failure of electrophoresis to separate albumin B from  $\alpha_1$  globulin necessarily introduces a systematic error into this estimate.

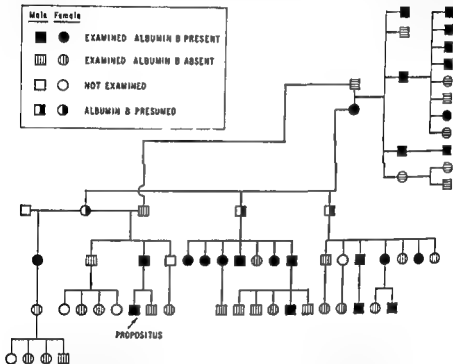


Fig 45-1 Genealogy of double albumin anomaly (Redrawn after Earle et al [40])

The authors feel that a correction for the average amount of  $\alpha_1$  globulin would make the quantities of the two albumins virtually identical in all their cases. As yet no study of the relative turnover rates of the two albumins has been effected.

### Genetics

The pattern of heredity may be fairly clearly defined from the accounts of double albumin anomaly which have been published. The genealogy of the family studied by Earle et al is reproduced in Fig 45-1. Since there is no known case in which the anomaly was found in the offspring of unaffected parents, it may be presumed that only the offspring of an affected parent are exposed to genetic risk. In the family studied by Earle et al, 28 of 46 individuals at risk are affected; in the other 5

hundreds 13 of 18 have the anomaly. When one remembers that there are necessarily 6 affected propositi it may be seen that the probability of inheriting the trait from an affected parent is not significantly different from 0.5. These data are interpreted by J. Lurie et al. as consistent with the hypothesis that there are two genetic loci controlling the synthesis of albumin that these loci are independent and that in the heterozygote there is equal production of normal albumin and albumin B. These authors consider that the anomaly is transmitted as a codominant characteristic both the abnormal and the normal genes being fully expressed. The homozygotic abnormal situation has not been encountered to date. Whether or not such a homozygotic individual would suffer from some clinically detectable disease cannot be predicted on the basis of presently available information.

### AN ALBUMINEMIA

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Bennhold Leters and Roth [42] first described a pair of siblings from whose plasma albumin was absent. Subsequent communications from the clinic at Tübingen [43-44] have amplified the original description and presented the results of a variety of biochemical investigations. Recently three more analbuminemic subjects have been reported, two in the United States and one in Switzerland [45-47]. The former two patients have been under observation since 1952 and 1956 respectively; the latter was only recently discovered. Pending the identification of more individuals with this disorder, the description of the disease must be based on this small series and generalizations must be regarded as tentative.

#### *Clinical Features*

The most striking feature of analbuminemia is the relative paucity of symptoms and signs. Edema, usually of mild degree, has been noted in all cases but one and has been relieved by the administration of albumin in the two individuals who received full replacement doses. The significance of edema cannot be assessed in the patient reported by Shetlar et al. [47] because of the coincident occurrence of congestive heart failure. Two subjects [44-45] have received replacement therapy with large amounts of exogenous human serum albumin and have noted an increased sense of well-being which may be taken as evidence of a preexisting mild malaise. The two young male patients, however, feel well enough to carry on heavy manual labor [43-46]. Bennhold and his colleagues [42] observed that their two patients with analbuminemia had a moderate degree of arterial hypotension, a feature which they considered to be an adaptation which helps to prevent excessive edema for

mation Of the three patients reported recently, one has normal blood pressure [50] one has a possibly slightly reduced value [46] and one [47] having aortic insufficiency cannot be considered in this respect

Associated disorders or abnormalities are present in several of these analbuminemic subjects, but these features each existing in only one case, cannot be considered an integral part of the picture of albumin deficiency The patient reported by Shetlar et al [47] suffers from rheumatoid arthritis of severe degree and from aortic valvular insufficiency leading to congestive heart failure He had sought medical aid for these conditions, and the analbuminemia was an incidental discovery The patient studied by Bartter and collaborators [50] suffers from the deformities typical of progressive lipodystrophy and has required surgical treatment for varicose veins The subject seen by Beck and Dorta [46] gave a history of cranial injury in childhood and subsequent convulsive episodes, he also was noted to have atrophic testes and a mild degree of feminization He had developed a dermatitis from handling cement and had sought medical attention for that disorder

The generally good health enjoyed by these patients in spite of their analbuminemia makes it impossible to assign an age of onset to the condition In four of the five subjects the anomaly was documented during young adult life and three of the five had been followed for over 6 years with no essential alteration in the condition It therefore seems likely that analbuminemia has been present since birth although the possibility that it is an acquired defect cannot be eliminated

### *Biochemical Aspects*

The term *analbuminemia* implies complete absence of serum albumin from the blood of these individuals The available evidence suggests that traces of albumin may be found by suitably exact methods but the quantities are so small that it seems justifiable to retain the present nomenclature The most thoroughly investigated subjects, from this point of view, are those at Tübingen In each of these, electrophoretic examination of serum revealed no albumin One subject was then treated with exogenous albumin the persistence of which makes other, more refined investigations impossible The other patient however, received no replacement therapy and has been reinvestigated more recently Lohss and Kallee [48] report the use of an elaborate procedure involving radioiodination immunologic precipitation and electrophoresis with the conclusion that if albumin is present the concentration must be less than 10 mg per 100 ml Later however Lohss and Ott [49] succeeded in demonstrating the presence of traces of albumin by immunoelectrophoresis Virtually identical findings are reported in the case of Beck and Dorta [46] although no albumin peak can be discerned on electrophoretic examination of whole serum immunoelectrophoresis in the

presence of an antialbumin serum reveals the presence of a trace of albumin. The case reported by Shetler et al. [47] was found to have less than 0.1 gm albumin per 100 ml serum with good agreement between results by electrophoretic and immunochemical methods. The case reported from the National Institutes of Health [48] had unfortunately been treated with exogenous albumin before any accurate measurements of serum albumin concentration were made.

The deficiency of serum albumin in analbuminemia can be attributed to failure of albumin production, not to an exaggerated rate of removal of albumin from the plasma. The disappearance of exogenous albumin administered to these subjects is indeed abnormally slow in the three instances in which it has been studied [44-47, 50]. In two instances the rate of removal of  $^{125}\text{I}$  labeled albumin has also been shown to be delayed [44, 45]. The failure of albumin production is not associated with other evidence of impaired liver function. The other plasma protein fractions are not decreased and in two cases [45, 47] liver biopsy has been carried out without the discovery of significant morphologic abnormalities. Beck and Dorta [46] performed a liver biopsy on their patient and assayed the tissue for a variety of enzymes. They noted a marked reduction in the activities of lactic malic and isocitric dehydrogenase and also decreased activity of glutamic-oxalacetic transaminase. The variability of their normal values for transaminase, however, is so great that this last finding is not clearly abnormal. They do not comment on morphology of the liver tissue.

Certain other biochemical abnormalities observed in the patients with analbuminemia may be considered to be effects, direct or indirect, of the lack of albumin. The concentration of serum globulins averages slightly above normal in the group, although no one component is strikingly increased. The results of laboratory procedures which depend upon serum protein concentrations (cephalin flocculation, thymol turbidity, Takata-Ura test) may be abnormal. The erythrocyte sedimentation rate is also uniformly elevated. Serum calcium concentration is reduced and there is a considerable increase in serum cholesterol, which was found to be as high as 610 mg per 100 ml in one instance [46] and over 400 mg per 100 ml in another [50]. In the two patients who have received adequate albumin replacement, these abnormalities have been corrected.

It is hardly possible to say that all the biochemical functions of serum albumin have been described. As the chief contributor to the colloid osmotic pressure of the plasma, albumin fulfills a role in the maintenance of physiologic relationships between intravascular and extravascular fluid volumes. Its vehicular function, suspected long ago by Bennhold [51], has been emphasized by recent studies of the binding of unesterified fatty acids by albumin [52] and the demonstration of the important role of this complex in the transport of fatty acids [53]. The patients with

analbuninemia are of great interest in so far as they demonstrate conclusively that neither these, nor any functions of albumin yet to be discovered are essential for life

### Treatment

Analbuninemia can be corrected in only one way—the infusion of sufficient human albumin to repair the defect (It is to be hoped that cases discovered in the future will be studied exhaustively before albumin

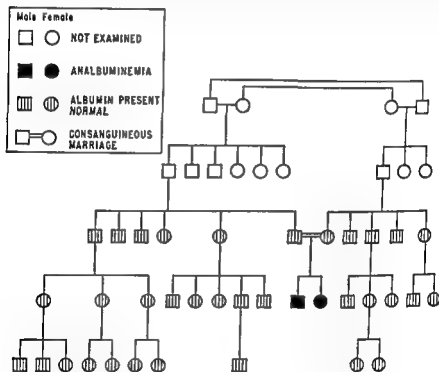


Fig 4-2 Genealogy of analbuninemia (Redrawn after Bennhold [49]) Symbols are the same as in Fig 45-1

supplementation is begun) The quantity of albumin required to maintain a reasonably normal blood level is in the range of 25 gm per week because of the slow degradation in these persons This amount can safely be tolerated in one infusion unless some associated condition presents a contraindication The advisability of instituting albumin treatment is not established on the basis of existing knowledge The mildness of the symptoms the apparently good prognosis and the availability of alternative means for the control of edema may be taken as arguments against the recommendation of costly and time consuming albumin treatment On the other hand the patients so treated appear to feel better and their

hypercholesterolemia which might contribute to the development of premature atherosclerosis is thereby corrected

### *Genetics*

A discussion of the pattern of inheritance of analbuminemia is not warranted at this time, since there are insufficient data to support any conclusion. There is no known example of transmission of analbuminemia from parent to offspring and the belief that it is a genetically determined anomaly is based solely on the existence of the condition in two siblings. The genealogy of these subjects has been published by Bennhold [43] and is reproduced in Fig 4a-2. It may be seen that these patients are the offspring of a consanguineous marriage so that there is a good possibility of an autosomal recessive pattern of inheritance. Beck and Dorta [46] suggest that there may also be some inbreeding in the ancestry of their patient although consanguinity between his parents was not known to exist. No other cases of analbuminemia have been found among his relatives [46] nor among the available relatives of the two American patients [47, 50].

## AGAMMAGLOBULINEMIA

### *Historical Aspects*

This disorder which has received considerable attention over the past 8 years was first described by Bruton in 1952 [54]. The pertinent literature has been reviewed by Good and Zak [55] and by Gitlin, Gross, and Janeway [56]; the latter presentation covers also the metabolism of this protein fraction and other diseases associated with abnormal serum concentrations. Agammaglobulinemia is customarily subdivided into congenital and acquired types. The former exists as the name implies from birth but is not manifest until passively transferred maternal  $\gamma$  globulin has been metabolized. Acquired agammaglobulinemia may develop at any age and in either sex; the distinction between the two forms in an individual patient is frequently impossible.

### *Metabolic Defect*

The metabolic defect in agammaglobulinemia consists of failure of  $\gamma$  globulin synthesis. The rate of degradation of  $\gamma$  globulin has been studied both with isotopic tracers [57-59] and by following the loss from the plasma of therapeutic doses of passively infused  $\gamma$  globulin [6, 50, 59]; it is not increased. Failure of antibody synthesis is not complete, however. In almost every case Gitlin et al [56] and Good and Zak [55] have been able to demonstrate small amounts of  $\gamma$  globulin by immunochemical procedures. In four cases studied at the National Institutes of Health [6] the serum contained small amounts of electrophoretically demon-

strable  $\gamma$  globulin. For this reason the term *agammaglobulinemia* is a misnomer but it is sanctioned by common use.

Agammaglobulinemia differs from an albuminemia in one important respect. While the liver in an albuminemia is morphologically intact the tissues of agammaglobulinemic subjects show a great deficiency or total absence of plasma cells. In addition, the lymph nodes are unusual in that they contain fewer lymphocytes than normal, and show ill-defined primary follicles [55, 60]. One may therefore suspect that the primary lesion in agammaglobulinemia is not solely a failure of the synthesis of one specific protein but that in some way it prevents the formation or maturation of the cells which should produce the protein. The condition might be considered closely allied to the familial structural malformations that have long been known to the anatomist.

### *Clinical Features*

The clinical manifestations of agammaglobulinemia are secondary to the failure of antibody synthesis and the resulting susceptibility to infection. Gitlin et al. [56] state that in their experience immunity to viral infections is not impaired but that the infections of agammaglobulinemic children are of bacterial origin. An interesting consequence of the lack of antibodies is that affected patients may not reject tissue homografts. Good and Varco [61] demonstrated the acceptance of a skin homograft in an agammaglobulinemic boy and this principle was employed therapeutically by Martin et al. [62] who transplanted lymph node tissue into an agammaglobulinemic adult. Subsequently there was immunochemical evidence of some renewal of antibody synthesis. Gitlin et al. [63] have pointed out the surprising fact that agammaglobulinemic subjects frequently develop the manifestations of rheumatoid arthritis or less often other collagen diseases. The significance of the occurrence of these conditions in patients who ought to be incapable of developing hypersensitivity is not yet known.

### *Diagnosis*

The diagnosis of agammaglobulinemia is established on the basis of chemical examination of the serum for  $\gamma$  globulin. Electrophoresis and immunochemistry are the methods most commonly employed for the definitive test. The condition should be suspected in any patient especially a male infant who manifests frequent bacterial infections and whose low resistance cannot readily be explained on any other basis. Since many such patients have repeated respiratory infections they may develop chronic pulmonary disease before the underlying condition is recognized. It is frequently possible to rule out agammaglobulinemia by the demonstration of normal blood type agglutinins. If these are absent in any but a type AB subject further examination of the serum



is indicated. Good and Tak [33] however note that an occasional agammaglobulinemic patient may have anti B agglutinin in his serum.

### Therapy

The accepted treatment for agammaglobulinemic patients consists of passive infusion of pooled normal human  $\gamma$  globulin combined with antibiotics to suppress infections. Present knowledge does not permit the prescription of a standardized program of therapy; treatment must be individualized. The antibiotics to be employed depend on the infecting organisms and their sensitivities.  $\gamma$  globulin should be administered intramuscularly in sufficient dosage to maintain a reasonable plasma level. No single therapeutically effective level can be established with certainty; the proper dosage must be ascertained empirically for each patient. A plasma  $\gamma$  globulin concentration of about 0.3 gm per cent is a reasonable initial goal; its achievement in an average 70 kg subject having no endogenous  $\gamma$  globulin will require a priming dose on the order of 25 gm protein. Maintenance of this level will require the administration of approximately 4 gm per week. The poor prognosis of untreated agammaglobulinemia can be considerably improved by careful management.

### Genetic Aspects

The acquired form of agammaglobulinemia is a sporadically occurring disease without known familial tendency. The congenital form on the

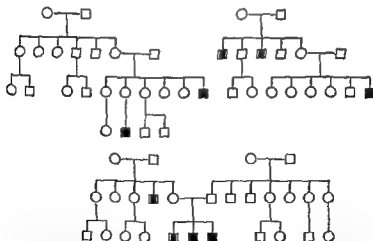


Fig. 4-3. Genealogy of congenital agammaglobulinemia. (Taken after G. H. L. [33]) Solid square = agammaglobulinemic male; open square = presumably affected—early death with history of recurrent severe bacterial infections. Open circle = presumably normal. The authors do not specify how many of these subjects were examined professionally.

other hand, appears to follow a definite genetic pattern. Diagnostic problems make the study of this condition difficult, since the disease may not be differentiated simply from physiologic hypogammaglobulinemia of infancy, on the one hand or from acquired agammaglobulinemia on the other. Nevertheless it is apparent that the congenital disease shows an overwhelming preponderance in the male sex. The few cases reported in female infants may be early examples of acquired agammaglobulinemia. Gitlin, Janeway, Apt, and Craig [63] have diagrammed three kinships in which are found five proved and four presumed cases of agammaglobulinemia. These genealogies are reproduced in Fig 45-3. In all instances the relationship between affected individuals is that of brothers or nephew and maternal uncle. Occurrence of cases in brothers is noted by Good and Zak [57] and in cousins by Porter [64]. The observations give strong support to the belief that congenital agammaglobulinemia is inherited as a sex linked recessive characteristic.

### OTHER INHERITED VARIATIONS IN PLASMA PROTEIN PATTERNS

Recent investigations utilizing the starch gel electrophoresis procedure described by Smithies [65] have brought to light several other variations in plasma protein patterns which appear to be inherited characteristics. None of these anomalies appears to be associated with any disease. Smithies and Walker [66] showed that normal human serums could be divided into three types on the basis of differences in the patterns obtained by their method of analysis. It soon became clear that the protein fractions on which this differentiation was made were the previously described haptoglobins, normal constituents of the serum which have the peculiar property of binding hemoglobin. These haptoglobin types have proved to be hereditary characteristics but the precise genetic mechanism of their transmission is not clear. An interesting hypothesis that might explain the findings is offered by Allison [67]. Individuals whose serum contains no haptoglobin have also been encountered [68] but it has not yet been shown that the defect has a genetic basis.

Several recent reports describe the findings of familial variants in the  $\beta$  globulin components revealed by starch gel electrophoresis. Smithies [69] found evidence for the occurrence of three types of  $\beta$  globulin and a recent communication by Harris, Robson, and Smiraglio [70] increases the number to five. All subjects so far reported have in their serum the  $\beta$  globulin designated C and in most individuals this protein predominates. In a number of Negroes and Australian aborigines however a slower moving protein designated D by Smithies [69] comprises about half the  $\beta$  globulin. In a few Caucasians a roughly equal amount of a faster moving component called B has been encountered. That these

variants are hereditary is shown in genealogies given by Smithies [69] and by Harris et al [70]

Fraser Harris and Robson [71] have reported the presence in one family of a plasma protein not usually seen in human plasma. The nature and significance of the component have not been elucidated but it appears not to have any detrimental effect on the members of the family who have it.

Finally, mention should be made of the occurrence in one small family group of an abnormally great thyroxine binding capacity of the serum  $\alpha$  globulin. This anomaly recently reported by Bierswales and Robbins [72] seems to be of clinical significance only because it results in an unusually elevated protein bound iodine value in euthyroid subjects. In one individual discovered by Tanaka and Starr [73] an unusually low protein bound iodine level seems to be related to the absence of thyroxine binding protein. There is as yet no evidence for a familial tendency or genetic basis for this defect.

### OTHER HYPOPROTEINEMIAS

Familial defects in plasma protein constituents are either responsible for or closely associated with several diseases discussed in other chapters of this book (Chaps 25, 30, and 44). In addition there is the hitherto poorly defined entity of idiopathic hypoproteinemia. Many such cases have been shown to have a defect allowing loss of macromolecules from the plasma into the gastrointestinal tract. This defect can be conveniently demonstrated with  $I^{131}$  labeled polyvinylpyrrolidone [74]. Actual loss of protein into the lumen of the stomach has been well demonstrated in one case studied by Citron, Sterling and Halsted [75]. The hypoproteinemia resulting from this protein losing gastroenteropathy should not at present be considered a biochemical lesion and no hereditary trend has yet been demonstrated.

An unusual family several members of which had abnormal serum protein patterns by electrophoresis was described by Homburger and Petermann [76]. It seems unlikely that the situation described is actually a hereditary biochemical anomaly since the manifestations were so varied and since many of the cases had protein concentrations which differed only slightly from the normal. The more severely affected individuals may actually have had protein losing gastroenteropathy if so they will comprise the first family group known to be so affected.

### SUMMARY

1. A number of variations in plasma protein patterns are known to be inherited characteristics. Some involving the absence of a protein are

associated with disease. Other conditions involving only a minor change in the chemistry of the protein while demonstrable by examination of the serum, do not produce any deleterious effects. The important hereditary protein anomalies are as follows:

2. Analbuminemia is an extremely rare condition characterized by failure of albumin synthesis. The affected subjects have mild edema but are otherwise well. There is virtually no albumin in the serum, and laboratory tests that are affected by serum protein concentrations are abnormal. Albumin replacement therapy is satisfactory and probably beneficial. The mode of inheritance has not been established, but the condition is probably transmitted by a recessive gene.

3. Agammaglobulinemia is characterized by a marked reduction but usually not total absence of  $\gamma$  globulin. Since the subjects are unable to form antibodies, they are unusually susceptible to bacterial infection, the consequences of which dominate the clinical picture. Antibiotic and  $\gamma$  globulin replacement therapy are helpful. Agammaglobulinemic patients lack plasma cells, the normal source of antibodies. The clinical disease may be acquired or congenital. The congenital form appears to be transmitted as a sex-linked recessive characteristic.

4. There are several conditions characterized by minor alterations in the chemistry of one or another plasma protein. These variants are detected by electrophoresis on paper or starch gel. None is associated with illness. This category includes double albumin anomaly and the genetically controlled variants in  $\beta$  globulins and haptoglobins.

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## Chapter 46

### Acatlasia\*

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James B. Wyngaarden and R. Rodney Howell

Acatlasia is a rare hereditary condition presently recognized in man only among the Japanese [1-5] though also known to occur in certain breeds of dogs [6] and guinea pigs [7]. The disorder was first described in 1949 by Takahara and Miyamoto. Three years earlier Takahara, a Japanese otorhinolaryngologist, noted that the operative field in a maxillary sinus from which he was removing a putrid tumor turned brown black upon the addition of hydrogen peroxide and did not foam. He also noted that blood from this patient turned dark brown on contact with peroxide. Investigation revealed that her blood lacked catalase and that several of her family members had the same defect.

The disorder was initially designated *acatalasemia* but since it is now known that catalase is absent from liver, muscle, and bone marrow as well as from the blood, the designation *acatalasia* [8] is preferred and will be used in this chapter to specify the entity. The term *acatalasemia* will be employed descriptively only.

### CLINICAL SYNDROME

Thirty-eight cases of acatalasia have been recognized in 17 Japanese families [4, 5]. About one-half have exhibited a peculiar and characteristic oral gangrene. In these patients gum and mouth infections occurred usually before age 10, and small ulcerations of the gums were observed as early as the second or third year of life. In some patients these ulcerations receded but were followed by new lesions which encroached upon the dental alveoli, thereby loosening the teeth. After the teeth fell out or were extracted, healing frequently occurred but in some instances gangrene with sequestration ensued. Extensive erosions into the maxillary

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sinus or the mandible sometimes led to development of putrid granulating tumors. Surgical intervention and bone grafting were then required. All degrees of ulceration occurred from minor erosions which healed readily to extensive oral gangrene. At times ulceration and necrosis involved the tonsil or the tongue. Recurrent episodes of gangrene at various sites in the alveoli with intermittent healing have continued for as long as 10 years [3]. Except for the oral lesions and fever in the presence of infection physical examinations were negative. Leukocytosis was not reported although mild anemia was observed. The absence of severe anemia indicates that the synthetic defect is not primarily one of heme elaboration. Treatment was directed toward excision of the gangrenous tissue and eradication of oral infection. After all teeth were removed and lesions had healed many patients were permanently free of symptoms.

*Illustrative Case (from Takahara [1])*

An 11 year-old girl presented with pain in her mouth. Three years earlier small ulcers had begun to appear on her gums. Some healed spontaneously but they were soon succeeded by others which spread to involve the dental alveoli thus loosening the teeth. When the teeth fell out or were removed healing occurred. After extraction of a tooth some weeks prior to her admission a sequestrum developed at the site of the tooth. Gangrene followed and progressed despite medical treatment. The right cheek swelled causing severe pain and a fever of 101 F developed.

The patient was a pale girl with greatly swollen and slightly reddened cheeks. The gums about her remaining teeth were gangrenous. Erosion of the right maxilla extended into the maxillary sinus and nasal cavity and produced a foul odor. Aside from a slight fever the physical examination was negative. Hemoglobin was 65 per cent of normal, red blood count 3,450,000 per cu mm. Erythrocyte sedimentation rate was 35 mm per hr. Bleeding and clotting times were normal and serologic examination was negative.

The right nasal cavity contained a foul friable granulating tumor. A biopsy specimen showed only chronic inflammation. Following radical removal of the tumor and curettage hydrogen peroxide was applied to the operative site whereupon the cavity in the maxilla and the surface blood immediately turned brownish black. Irofs or Takahara supposed silver nitrate had been applied by mistake promptly washed the wound with saline solution and applied hydrogen peroxide from a new bottle only to observe a repetition of the color change. He then noted that the black color was caused by blood oozing from the wound and that very few bubbles had formed.

The postoperative course was complicated by three recurrences of sequestrum formation necessitating curettage but the lesions healed completely in 2 months. One year later ulceration recurred at the site of the extracted teeth and despite treatment again progressed to development of gangrene.

After a month of therapy which included surgical drainage the disease was checked. Nine months later another recurrence with sequestration was successfully treated with penicillin. Subsequently the patient's remaining teeth were pulled whenever ulcers appeared in her gums. At the age of

15 she was edentulous but fully developed and apparently well. Investigations revealed absence of catalase in her erythrocytes and oral mucosa.

Further investigations revealed that three of five siblings of the patient also lacked catalase in their blood. Two of them had the same oral disease as the patient. A further disclosure was that the parents were second cousins.

### CATALASE STUDIES

When hydrogen peroxide solution is dropped into normal blood, gas is evolved and the color does not change. When hydrogen peroxide was dropped into the blood of the patient described above it blackened immediately and gas did not appear [1]. Blood from three of five siblings gave an identical response. These observations suggested to Takahara that blood catalase was deficient. Using Warburg's manometric method [9, 10], he established that catalase activity was indeed grossly deficient in blood and also in oral tissue obtained from these patients. The results of his initial studies on five patients are shown in Table 46-1, they indicate a virtual absence of catalase activity in both types of tissue in these subjects. Subsequently, confirmatory results were obtained in studies of blood of additional patients by a permanganate titration method [11, 12]. The values in all patients suspected of lacking catalase by the qualitative test approached zero when analyzed by quantitative methods. Blood peroxidase levels were normal in all patients [1].

When a small quantity of  $H_2O_2$  was added to acatalasemic blood in a

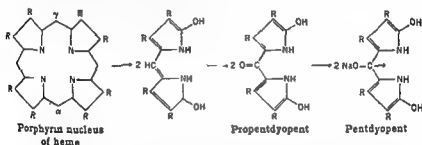
TABLE 46-1 CATALASE ACTIVITY IN BLOOD AND MUCOUS MEMBRANES OF ACATALASEMIC AND NORMAL SUBJECTS

| Case | Sex | Age, yr | Material examined            | $Q_{30}$              |                 |
|------|-----|---------|------------------------------|-----------------------|-----------------|
|      |     |         |                              | Acatalasemic subjects | Normal subjects |
| 1    | F   | 16      | Blood from ear lobe          | 7                     | 4,500           |
| 2    | M   | 18      | Venous blood                 | 5                     | 4,500           |
|      |     |         | Blood from ear lobe          | 6                     | 4,500           |
| 3    | F   | 8       | Blood from ear lobe          | 10                    | 4,500           |
|      |     |         | Normal oral tissue           | 0                     | 750             |
| 4    | F   | 14      | Blood from ear lobe          | 10                    | 4,500           |
| 5    | M   | 17      | Venous blood                 | 0                     | 4,500           |
|      |     |         | Erythrocytes of venous blood | 8                     | 12,000          |
|      |     |         | Normal oral tissue           | 0                     | 750             |
|      |     |         | Oral ulcer                   | 0                     |                 |
|      |     |         | Tissue of ethmoidal sinus    | 0                     | 450             |
|      |     |         | Nasal polyp                  | 0                     | 200             |

$Q_{30}$  = cubic millimeters of  $O_2$  generated at 38°C in 30 min per mg tissue (dry weight) [8, 9].

SOURCE: S. Takahara [1].

test tube the blood remained black for a long time. If an excess of peroxide was added the blood began to lose its dark color in 20 to 30 min and became light reddish brown then lemon yellow and finally colorless. This solution was shown to contain propentdyopent = colorless decomposition product of hemoglobin. When this solution was made alkaline with potassium hydroxide heated and reduced with sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) = beautiful rose color developed [1]. This rose colored compound previously named *pentdyopent* by Bingold [13-14] because of its absorption maximum at  $525 \text{ m}\mu$  is now known to be = dipyrrole [15]. The colorless precursor named *propentdyopent* by Fischer and von Döbeneck [16] is a closely related substance. The probable structures of these compounds [17] and their relationship to hemoglobin are shown.



Takahara next ingeniously correlated the pentdyopent reaction observed in the test tube with biologic events involving bacteria isolated from the oral cavities of affected patients [1]. He prepared blood agar plates with normal and acatalasemic blood and planted hemolytic streptococci or pneumococci type I obtained from an acatalasemic subject on these plates. These two organisms were the predominating organisms in the mouth lesions of most of the subjects with acatalasia. The colonies grew equally well on both types of agar but on the acatalasemic blood agar wide transparent rings appeared around the colony instead of hemolytic rings growing larger with time. These colorless zones showed a positive pentdyopent reaction! This study showed that peroxide was produced by the = organisms in quantities sufficient to destroy hemoglobin in the blood agar lacking catalase.

Kaziro and associates [8] analyzed selected tissues from an asymptomatic adult patient with healed oral lesions and known acatalasemia. They found no catalase activity in biopsy specimens of liver, muscle and bone marrow although catalase activity was demonstrated in these tissues from = control subject. Although this does not constitute exhaustive proof that all viscera are devoid of catalase in patients with acatalasemia these results and those of Takahara showing absence of catalase in oral mucous membranes of such patients provide strong evidence for this likelihood. Kaziro et al interpreted their data as evidence for a

"constitutional acatalasia" and proposed the term *anerymia catalasica* for this genetic anomaly. The simpler term *acatalasia* is here proposed as nomenclologically preferable to *acatalascemia* and as an etymologically acceptable designation for this genetic anomaly.

## CATALASE

Thénard [18] noted in 1818 that both plant and animal tissues degrade hydrogen peroxide, a substance he himself had discovered some years earlier. Loew [20] in 1901 gave the name 'catalase' to the substance responsible for this degradation. In 1923 Warburg [2] suggested that catalase is an iron-containing enzyme because it is inhibited by cyanide. Evidence that its prosthetic group is hematin was offered in 1930 by Zeile and Hellstrom [21]. Catalase was crystallized from beef liver in 1937 by Sumner and Dounce [22] who reported an iron content of about 0.1 per cent and emphasized that the prosthetic group is heme or a closely related substance.

### The Prosthetic Group

Catalase activity is widely distributed in mammalian tissue but is most abundant in liver, kidney, and erythrocytes [23-24]. Catalase has been crystallized from these three tissues of a number of species [24-26], including man [26-28], as well as from bacteria [29]. Animal catalase is an iron porphyrin protein with a molecular weight of 220,000 to 250,000 containing four porphyrin nuclei and four atoms of iron per molecule (0.09 per cent) [30-31]. Several authors [32-33] believe that different catalases exist containing 4, 3, 2, 1, and 0 hematin groups and 0, 1, 2, 3, and 4 verdohematin groups. However, Bonnichsen has suggested that the oxidized porphyrin groups are post mortem artifacts or changes occurring during isolation and has reported that human liver [26-27] and erythrocyte [34] catalases have no verdohematin groups. In human catalase as in beef liver catalase the porphyrin nucleus is the ferric complex of protoporphyrin IX (corresponding to etioporphyrin isomer type III) (protohematin) [35]; it is identical with that found in horse radish peroxidase, methemoglobin, and metmyoglobin and is similar to that of ferri cytochrome C. The differences in chemical nature and physiologic role of these iron porphyrin proteins are attributed to differences in the protein moieties and in the character of linkages between protein and prosthetic group. The structure and biosynthesis of protoporphyrin IX are discussed in Chap. 30.

The catabolism of the heme of catalase is apparently identical with that of hemoglobin and ultimately yields bilirubin [36]. The ferriprotoporphyrin of catalase is easily removed from the apoenzyme by acid acetone recombination has not yet been achieved [37]. The enzyme gives

pectroscopically characteristic derivatives with cyanide fluoride azide and other anions and with its substrate hydrogen peroxide

### *The Apoenzyme*

Theorell and Åkesson [38] determined the nitrogen distribution and basic amino acids of horse liver catalase Bonnichsen [34] compared horse liver and blood catalases and found them to contain identical quantities of histidine arginine lysine glutamic and aspartic acids and amide nitrogen Immunologically there was no difference between them and

TABLE 46- AMINO ACID COMPOSITION OF BOVINE LIVER CATALASE

| Amino acid    | Gm/100 gm protein | Moles/mole of catalase |
|---------------|-------------------|------------------------|
| Serine        | 3.10              | 67                     |
| Glycine       | 3.26              | 98                     |
| Alanine       | 4.27              | 108                    |
| Threonine     | 3.31              | 63                     |
| Valine        | 6.14              | 118                    |
| Methionine    | 2.83              | 43                     |
| Cystine       | 1.12              | 10                     |
| Proline       | 4.54              | —                      |
| Leucine       | 8.60              | 148                    |
| Isoleucine    | 3.84              | 66                     |
| Phenylalanine | 4.04              | 104                    |
| Tyrosine      | 6.80              | 85                     |
| Tryptophan    | 3.58              | 40                     |
| Aspartic acid | 11.40             | 193                    |
| Glutamic acid | 9.43              | 149                    |
| Lysine        | 3.88              | 152                    |
| Arginine      | 4.91              | 103                    |
| Histidine     | 7.70              | 122                    |
| Ammonia       | 2.16              | —                      |
| Heme          | 1.03              | —                      |
| Total         |                   | 1,748                  |

SOURCE: From G. Schnuchel [39].

in the ultraviolet their absorption coefficients were the same. Horse blood catalase differed from human blood catalase immunologically.

Recently Schnuchel [39] published a complete amino acid analysis of crystalline bovine liver catalase (Table 46-2). It contains 18 amino acids and 1,748 amino acid residues and more histidine and lysine than were reported for horse liver catalase [34, 38]. Thus regardless of the interpretation of differences in the porphyrin prosthetic groups it is clear that catalases of different species vary in the composition of the apoenzyme.

### *Tissue Catalases*

Theorell and associates [40] investigated the origin of liver and blood catalase in guinea pigs using  $Fe^{55}$  and  $Fe^{59}$ . Activity in liver ferritin

initially exceeded that in liver catalase but approached equal labeling at about 10 days and thereafter declined in parallel. Blood catalase in contrast showed a slower increase that ran parallel to the hemoglobin iron activity. They concluded that identical enzymes were being formed in different organs. The short life cycle of liver catalase suggested to Theorell [19] that the verdohematin prosthetic groups previously found in liver catalase [32, 33] may be normal intermediates in the destruction of catalase.

Additional evidence for independent origins of heme of liver and erythrocyte catalases is provided by studies of Schmid, Ligen, and Schwartz [41]. The administration of allylisopropylacetyl carbamide (Sedormid) to rabbits and rats causes simultaneously a marked and rapid fall in liver catalase activity, a great increase in liver porphyrin concentration and porphyrinuria. No significant change occurs in erythrocyte catalase activity, in hemoglobin concentration or in activity of liver cytochrome oxidase and succinic dehydrogenase. Studies with glycine-2- $C^{14}$  suggested that Sedormid blocks formation of catalase in liver; the porphyrin accumulations in liver and urine were viewed as possible consequences of this block. The observation that daily excretion of porphyrins and their pyrrole precursors exceeded the amounts of catalase heme normally found in liver may be a reflection of the extremely rapid turnover of liver catalase. It also suggests that catalase or a product of it may normally exert a feed back regulatory effect upon hepatic porphyrin synthesis.

Finally, levels of catalase activity of liver can be varied experimentally but those of erythrocytes remain constant under a variety of circumstances. Among the factors which selectively reduce catalase activity of liver or other viscera are starvation [42], iron deficiency [43], copper deficiency [43], castration (in male rats only) [44], adrenalectomy (in rats of both sexes enhanced by prior castration in the male) [44], endogenous [45] or transplanted [46] tumors, nitrogen mustard, carcinogens or hepatotoxins [47]. Elevations of liver catalase activity are produced in adrenalectomized rats by cortisone and in castrated male rats by testosterone. Both hormones are required for complete restoration of normal activity in castrate adrenalectomized male rats. Testosterone increased kidney and liver but not blood catalase activity in normal female mice [44].

In severe human iron-deficiency anemia there is no change in catalase activity per milliliter of erythrocytes although there is a slight decrease per million red cells [48]. If it is assumed that catalase and hemoglobin derive heme from a common source [40] these results indicate that catalase synthesizing mechanisms compete successfully for a limited amount of heme under conditions of iron deficiency. In iron deficiency in animals catalase activity of heart remains normal despite a decline in activity in other viscera [43].

Only one abnormal value of blood catalase activity was noted in 152 patients with a variety of disorders. Similar analyses in diseased animals gave only normal results [49]. Blood catalase is said to be elevated in acute malaria and normal in the chronic phase [50].

### CATALASE FUNCTION

#### *Decomposition of $H_2O_2$*

It was originally thought that catalase served as a physiologic safeguard to decompose  $H_2O_2$  which might otherwise accumulate within the cell. Bingold [51] suggested in 1933 that the role of erythrocyte catalase was to protect hemoglobin from oxidation by  $H_2O_2$ . It was shown that inhibition of catalase by hydroxylamine permitted rapid oxidation of hemoglobin to methemoglobin when peroxide was added. Keilen and Hartree [52] reasoned that the violent decomposition of  $H_2O_2$  in Bingold's experiments generated much  $O_2$  and maintained hemoglobin as oxyhemoglobin in which form its iron is resistant to oxidation. They demonstrated formation of methemoglobin despite the presence of abundant catalase activity when  $H_2O_2$  was generated slowly by a glucose oxidase system. Since this experiment simulated conditions *in vivo* they concluded that catalase does not function to protect hemoglobin.

#### *Peroxidatic Activity*

In 1936 Keilen and Hartree [53] demonstrated coupled oxidation of ethanol in the presence of a peroxide generating system (xanthine oxidase and hypoxanthine) and catalase. It is now well established that with high substrate concentrations of low molecular weight alcohols, formaldehyde or formic acid, and low concentrations of peroxide, catalase exhibits peroxidatic activity.

Chance [54, 55] has studied the mechanisms of the actions of catalase. The initial reaction involves formation of a complex between catalase and  $H_2O_2$  in which peroxide is bound to the ferric iron of heme.



The enzyme substrate complex can then react with a hydrogen donor—a peroxidatic reaction:



Or it can react with a second molecule of  $H_2O_2$ —a catalatic reaction:



The catalase-hydrogen peroxide complex has been identified spectrophotometrically in aerobically respiring cells of *Micrococcus lysodeikticus*. The steady state intracellular hydrogen peroxide concentration in these organisms was calculated to be  $10^{-8} M$  [57, 58].

### Physiologic Roles

Chance believes that catalase acts both catalatically and peroxidatically *in vivo* [56] and has pointed out [57] that the partition between catalatic and peroxidatic utilization of hydrogen peroxide will depend mainly upon the steady state peroxide concentration. The velocity of the catalatic reaction increases linearly with the peroxide concentration; the peroxidatic reaction is essentially independent of peroxide concentration. Catalase may thus act as a regulator of hydrogen peroxide concentration; small amounts of peroxide will saturate the peroxidatic function, larger amounts are decomposed in the catalatic reaction. The peroxidatic action of catalase requires only very low concentrations of peroxide, about  $10^{-6}$  M. Thus at a steady state concentration of  $10^{-8}$  M, as in *M. lyso deikticus* [55, 56], catalase is probably acting by a combination of the catalatic and peroxidatic pathways.

Since physiologically there may exist high concentrations of other acceptors and low concentrations of peroxide, it is conceivable that catalase serves primarily as a peroxidase in animal tissues and only secondarily as a defense against accumulation of peroxide. Indeed the catalatic reaction  $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$  may be viewed as merely a special case of a peroxidatic reaction where hydrogen peroxide serves both as substrate and as acceptor. Theorell [19] has proposed that both catalatic and peroxidatic enzymes be called *hydroperoxidases*, to emphasize that they have a common substrate  $\text{H}_2\text{O}_2$ .

Catalase is not absolutely specific and will act upon organic peroxides such as ethyl hydrogen peroxide under certain circumstances [58, 59]. Its rate of decomposition of hydrogen peroxide is the highest of any enzymatic rate studied to date. One molecule of catalase is capable of splitting 42,000 molecules of  $\text{H}_2\text{O}_2$  per second at  $0^\circ\text{C}$ .

Catalase is inactivated in 10 min at  $60^\circ\text{C}$  but is stable at  $4^\circ$ . It is inhibited by a large number of compounds, some of which do not affect other heme protein enzymes. In hemolyzates of normal blood, inclusion of 2,4-dichlorophenol to inhibit catalase permits demonstration of the peroxidatic action of methemoglobin [60] which will then result in rapid oxidation of uric acid (or other suitable acceptor) in the presence of peroxide. Hematin split from catalase by acid acetone will function peroxidatically but not catalatically in this system, demonstrating the essential contribution of the catalase protein to its catalatic activity [61].

### HYDROGEN PEROXIDE

The sources of hydrogen peroxide become important in consideration of the pathogenesis of the oral lesions of subjects with acatalasia.  $\text{H}_2\text{O}_2$  is produced by aerobic dehydrogenases—enzymes which transfer hydrogen



directly to molecular oxygen of which several are found in mammalian tissue. All the enzymes of this series employ flavin adenine dinucleotide (FAD) as coenzyme, and occur as cytoplasmic constituents. A number have also been identified in the insoluble particles of liver presumably the mitochondria. Peroxide formation then must be a normal occurrence in mammalian cells.

The important aerobic dehydrogenases of mammalian tissue are monamine oxidase, diamine oxidase,  $\beta$ -amino acid oxidase, L-amino acid oxidase, glycine oxidase, glycolic oxidase, xanthine oxidase, and uricase, although the last enzyme does not occur in human tissue.

That coupled peroxidatic reactions of the types discovered by Thurlow [62] and by Keilen and Hartree [59] may be of physiologic significance was shown by Heppel and Porterfield [63] who found sufficient hydrogen peroxide produced in rat liver homogenates to promote a readily measurable oxidation of nitrite to nitrate by catalase.

Aerobic dehydrogenases occur in bacteria also, and production of  $H_2O_2$  has been detected in several aerobic organisms which contain no catalase, such as pneumococci, *Bacillus acidophilus*, and certain streptococci [51].  $H_2O_2$  is presumed to occur in other aerobic or facultative anaerobic organisms also, but to escape accumulation because of the presence of catalase, which is known to exist in *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and others [51].

## PATHOGENESIS OF LESIONS OF ACATALASIA

Individuals with acatalasia show no signs of ill health other than mouth lesions. When all teeth are lost or removed, and the lesions treated, healing occurs and the individuals remain well thereafter. Some subjects with acatalasia are asymptomatic throughout their lives. These observations strongly imply that oral sepsis initiates the ulcerative, gangrenous process, and that individuals with acatalasia are vulnerable to attack by ordinarily innocuous bacteria of normal oral flora.

### Local Lesions

Takahara [1] reasoned that gums are apt to be injured during mastication, and that wounds in gums are suitable media for bacteria. The most commonly cultured organisms, hemolytic streptococci and pneumococci type I, are producers of hydrogen peroxide and lack catalase. In persons who lack catalase in blood and oral tissue, serious effects result, because hydrogen peroxide oxidizes blood reaching the lesion, thus depriving the infected area of oxygen and causing necrosis. This would favor multiplication of bacteria, increased production of peroxide, and oxidation of hemoglobin.

Additional factors may be involved [8]. Several other potential effects

of peroxide on tissue should be considered, such as possible actions upon sensitive heme proteins. Activity of catalase has been observed to decline in high concentrations of peroxide in the presence of a reducing substance, probably because of autocatalysis of oxidation of the protohematin groups to bile pigment hematin groups [33]. In the absence of catalase similar changes might well involve tissue peroxidases, cytochromes, and myoglobin. Production of metmyoglobin would render it incapable of binding  $O_2$  in muscle. Recently, even higher valence states of iron (ferryl myoglobin) [64], or production of free radicals of myoglobin through loss of hydrogen from the conjugated ring system of the porphyrin moiety [65] have been attributed to hydrogen peroxide.

Dixon [66] showed that catalase protects xanthine oxidase from destruction by the hydrogen peroxide which it forms. This may be an example of the well known susceptibility of flavin prosthetic groups to destruction by peroxide or it may illustrate a general effect of peroxide on proteins. Peroxide will oxidize sulfhydryl groups potentially all the way to sulfonic acid residues, and a large percentage of enzymes depend upon sulfhydryl groups for functional activity.

Peroxide is hemolytic [67]. It is also known to oxidize directly a number of compounds found in cells, e.g.,  $\alpha$  keto acids. Thus a large number of effects in addition to destruction of hemoglobin may be anticipated if tissue lacking catalase is exposed to hydrogen peroxide.

### *Systemic Effects*

About one half of the subjects with acatalasia are asymptomatic, and many of the others remain entirely well when mouth lesions have healed. Initially it was not known that tissue catalase was also absent and it remained possible that subjects with acatalasemia lacked catalase only in blood and in injured oral mucous membranes. Had this been so, these subjects would have had a catalase distribution like that of the duck, in which blood lacks catalase but other tissues have abundant activity of this enzyme [8]. With the demonstration of absence of measurable catalase activity of other tissues in an asymptomatic subject with acatalasemia it became clear that catalase was not an indispensable enzyme and that in its absence toxic levels of  $H_2O_2$  did not accumulate. Thus an experiment of nature has answered one important question and it is now clear that catalase does not serve as an essential physiologic safeguard. One might wonder whether subjects with acatalasia would show increased levels of methemoglobin in circulating erythrocytes but in one analysis such an increase was thought questionable [8].

Recently it has been shown that peroxidases are widely distributed in mammalian tissue [68] and presumably these enzymes utilize such peroxide as is produced as substrate for their oxidative functions. It will be recalled that peroxidase was normal in acatalasemic blood pre-

sumably representing peroxidatic activity of leukocytes [69-70]. Consumption of peroxide in such reactions apparently is quantitatively sufficient to prevent peroxide toxicity even in the absence of catalase unless infection with catalase free organisms supervenes.

## TREATMENT

Treatment of oral lesions has been highly successful in preserving life and in eventually controlling the progression of the necrotic changes. Surgical excision of granulating tumors, curettage, drainage and irrigation of septic areas, extraction of teeth and antibiotic therapy have been employed. Reconstructive surgery and bone grafts have been required. The direct application of crystalline catalase suspensions for control of acute lesions has been suggested [71]. The effects of transfusions of normal whole blood containing catalase would also seem worthy of evaluation.

## GENETICS

The 38 known cases of acatalasia occurred in 17 families living in various parts of Japan. In all but 3 families 2 or more siblings were affected. In 2 families as many as 4 siblings were affected. A history of consanguineous marriage was obtained from 16 families. Of the 38 cases 19 occurred in males and 19 in females. The first pedigree discovered containing 13 cases of acatalasia have been reported in detail [1, 2]. In each one (or more) consanguineous marriage was found a total of 9 in all. In 3 families both parents and children with acatalasia were living and were studied. Not a single parent had acatalasemia. Also in 1 family 12 of 15 children of 3 acatalasemic members were tested and none had acatalasemia. These findings virtually exclude dominant inheritance.

The findings of consanguinity, involvement of families at one sibling level only and equal sex distribution are suggestive of non sex limited recessive inheritance. In a study of 27 of 31 members of 5 sibships including known acatalasia subjects 13 acatalasemic and 14 normal members were found [1, 2]. If one excludes from analysis the 5 probands the finding of 8 acatalasemic subjects among 22 siblings is in satisfactory agreement with an anticipated incidence of 25 per cent homozygous abnormal in individuals from a nonlimited recessive inheritance.

Catalase assays have been done on the blood of 66 members of 5 families [72] by a modification of the method of von Euler and Josephson [73-74]. The distribution of values of activity units was clearly trimodal (Fig 46-1). In 10 subjects values were zero; in 30 they ranged from 1.48 to 2.89; in the remainder they were comparable to values in 206 control subjects which ranged from 3.91 to 7.10. Thus subjects exist in these

families who show intermediate values of catalase activity which do not overlap with values for the controls. Presumably these are 'carriers' heterozygous for the acatalasia trait. This investigation showed that (1) the parents of acatalasemic subjects are hypocatalasemic, (2) the

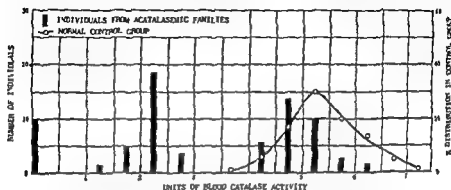


Fig 40-1 Distribution of catalase values for members of five acatalasia families and comparison with a percentage distribution curve of values from a normal control group (By permission of E. T. Vishimura et al and Science [2])

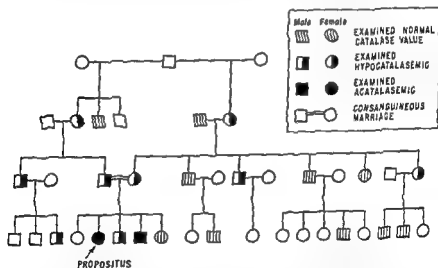


Fig 40-2 Genealogic relationships in an acatalasia family (Redrawn from E. T. Vishimura et al [2] Reproduced with permission of authors and publisher)

siblings of acatalasemic individuals may be acatalasemic, hypocatalasemic, or normal. (3) an acatalasemic parent has hypocatalasemic children when mated to a normal person. These findings and those of an expanded study recently published [7a] appear to document fully the earlier conclusions of Takahara and coworkers [2, 3] that acatalasia is transmitted by incompletely recessive monogenic inheritance. A typical genealogic diagram appears in Fig 40-2.

Allison Rees and Burn [6] noted variations in blood catalase activity in a large group of dogs of various breeds. In some dogs activity values were quite low and blood darkened on the addition of  $H_2O_2$ . Controlled breeding of these animals disclosed a simple mode of recessive inheritance for which a single pair of alleles was proposed. Heterozygous dogs showed values of blood catalase intermediate between those of homozygous animals and those of normal dogs. Tissue catalase values were not reported.

Radev [7] obtained similar genetic results in a study of guinea pigs with normal and low blood catalase activity values. Animals with low blood values were also found to have low catalase activity values in their livers. No signs of ill health were noted in either guinea pigs or dogs with low catalase activities.

## SUMMARY

1 Acatalsia is a rare hereditary disorder presently recognized only among the Japanese in which ulcerating gangrenous lesions of the oral cavity may be observed. The oral mucous membranes, erythrocytes, liver, muscle and bone marrow of affected individuals lack catalase activity.

2 The lesions appear to be caused by the destructive effects of hydrogen peroxide generated by catalase free bacteria growing within the mouth. In subjects with acatalasia hydrogen peroxide injures oral tissue possibly by destruction of structural and enzymatic proteins through oxidation of their sulfhydryl groups, by oxidation of heme proteins such as hemoglobin, myoglobin, peroxidases and the cytochromes and by oxidation of flavoproteins.

3 Acatalsia may be entirely asymptomatic and affected persons whose teeth are removed and whose lesions have healed may thereafter be entirely well. Catalase appears to be a nonessential enzyme except for the protection it affords against  $H_2O_2$  generated by bacteria.

4 Acatalsia is transmitted as an autosomal recessive Mendelian characteristic. Consanguinity has been found in most pedigrees of acatalasia. The distribution of blood catalase activity values in 66 members of 5 families was clearly trimodal and no overlap was observed. The intermediary values presumably defined individuals who were heterozygous for the acatalasia trait. Both parents, some siblings and all children of acatalasemic subjects were heterozygous by this criterion.

5 An apparently analogous disorder of catalase synthesis with similar genetic characteristics has been described in certain breeds of dogs and guinea pigs. Heterozygous dogs show blood catalase activity values intermediate between those of normal and homozygous animals. Guinea pigs with low blood catalase activity values also show low activity in liver

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## Appendix

### Methods of Coding and Filing Family Records

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Grace J. Yesley

The student of human genetics who is confronted for the first time with an extended pedigree or a collection of moderate sized pedigrees rapidly acquires a healthy respect for the problems inherent in ordering such data. Over a period of 15 years the Department of Human Genetics of the University of Michigan has developed a system of recording and coding pedigree data that has proved of convenience in the study of hereditary disease. This appendix constitutes the first description of that system. The system is composed of three principal sections (1) a numerically arranged *Kindred Folder File* (2) a *Surname Card File* and (3) a *Diagnosis Catalogue*.

#### KINDRED FOLDER FILE

By placing all information on a kindred (pedigree results of any examinations performed etc.) in a folder each kindred record may be easily separated from the records of other kindreds. As the number of kindreds on file accumulates it becomes advantageous to develop an efficient filing system so that (1) records of all kindreds which have a person or persons involved with some particular disease may be quickly taken from the files for examination and study and (2) it may be rapidly ascertained if a person or his family has already been seen at the Clinic.

By assigning a kindred number to each kindred the records may be filed numerically. Advantages of this system over an alphabetic system of filing are apparent to anyone who has worked with an alphabetic system and been faced with the problem of frequently having to shift almost all the records to make room for those new records which are continually being placed within the system.

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*Pedigree*

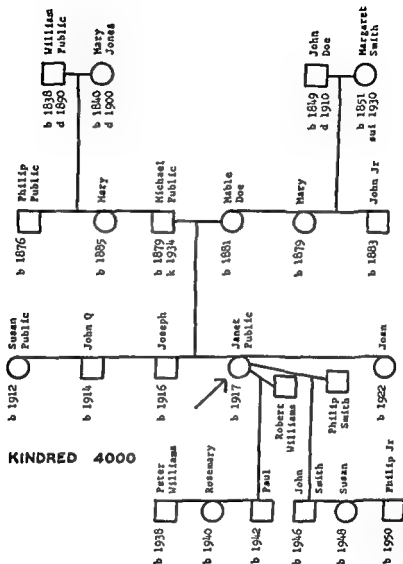
A pedigree is the backbone of any study of hereditary disease. It may be presented in a number of acceptable forms. The conventions employed at the Department of Human Genetics, University of Michigan Medical School, have proved to be useful and informative, and are accordingly described here. The members of each sibship are recorded in the order of their birth, with the eldest on the left and the youngest on the right, with a few exceptions which will be discussed later. Every pedigree has as a focal point (indicated by an arrow) the *propositus*, the person who initially brought the kindred to the attention of the physician or geneticist. The pedigree then is built around this *propositus*.

The male ancestors of the *propositus* and his relatives are drawn on the left and the female ancestors and her relatives on the right (Fig. 1). To facilitate the drawing and eventual reading of the pedigree, an exception is made to the general rule of arranging members of a sibship in their birth order with respect to the parents of the *propositus*: the male parent is drawn at the end of his sibship and the female parent at the beginning of her sibship, irrespective of birth order (Fig. 1). Each ancestral sibship is also drawn in this manner, with the male ancestors at the end of their sibships and the female ancestors at the beginning of their sibships. Sibship lettering starts with the sibship of the *propositus* (Sibship A) and ascends from left to right from that sibship through the sibships of the grandparents. Sibship letters A through G then have definite meaning, with Sibship B always referring to the father's sibship, D to the paternal grandfather's sibship, etc. (1). The letter H is always reserved for the sibship containing the offspring of the *propositus*. I, J, K, . . . W may be used for any ancestral lines beyond the grandparents which are of special interest.

At this stage, all sibships descending from the siblings of the ancestors and the siblings of the *propositus* are identified by the sibship lettering of their parents (the parents biologically related to the *propositus*) with one letter added. That is to say, sibships which are first-generation descendants of Sibship B would be labeled BA, BB, BC, etc., with Sibship BA being the offspring of the eldest member of Sibship B, BB the offspring of the second eldest, etc. By strict adherence to this coding system in all records, one may at a glance determine the exact genetic distance from the *propositus* of any relative whose sibship lettering starts with a letter from A to H [i.e., BA, BB, BC, etc. = paternal first cousins; CA, CB, CC, etc. = maternal first cousins; BAA, BAB, BBA, etc. = paternal first cousins once removed (offspring of paternal first cousins); DAD, DBA, DBB, etc. = paternal second cousins, etc.].

Other occasionally needed and therefore standardized codings are as follows:

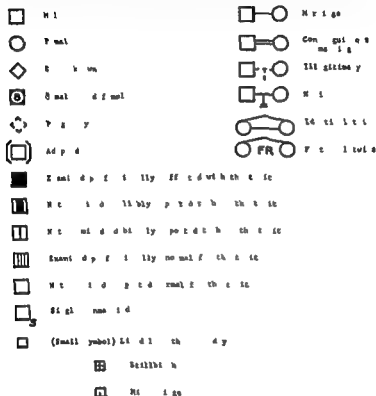
# KINDRED 4000



APPENDIX FIG 1 Hypothetic pedigree illustrating the method used for including names of biologic relatives and spouses birth and death dates

The names of all biologic relatives of the propositus are customarily written above the sibship line and the names of unrelated persons (spouses of relatives) are written below the sibship line. Birth and death dates are written below the symbol of each person.

Although every individual develops his own method of taking notes during interviews, there are in general two approaches to this problem.



APPENDIX FIG. 3. Symbols frequently used in drawing pedigrees.

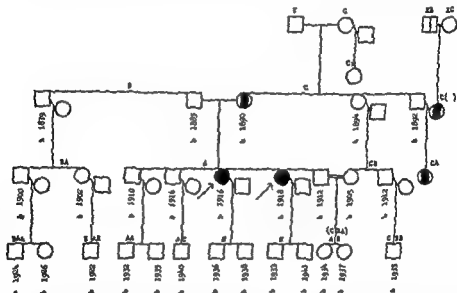
The family history may be taken in pedigree form, all the pertinent information being given on the pedigree, or in outline form, the data being jotted down by sibship in the sequence in which the pedigree is assembled. Figure 3 illustrates the standard shorthand symbols used in drawing pedigrees.<sup>1</sup>

The above steps provide the basic structure of the pedigree coding system with methods for handling the common problems. From this point on, the problems become more or less specific to the clinic physician or geneticist using the system, modifications of or additions to the

1 2 (subscripts) = half siblings ( $H_1$  = half siblings of  $H$ ,  $H_2$  = half-siblings of  $H$  and  $H_1$ , i.e., children of a third marriage by the parent in common to  $H$  and  $H_1$ .)

Y, Z = off-spring of per on adopted into the family (persons of little or no genetic interest in the study of that particular family but often valuable as informants)

At times one encounters a family in which consanguineous or affinous (relationship between persons through another marriage but not through

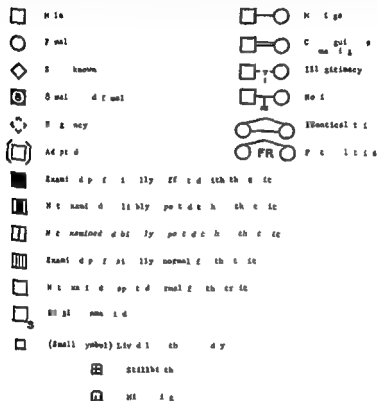


Appendix Fig 2 Hypothetic pedigree illustrating sibship lettering system

a blood line ) marriage has occurred. Often these relationships may be more readily illustrated if the siblings are drawn out of strict birth order. Consanguineous marriages are by convention indicated by a double marriage line. Figure 2 illustrates a consanguineous marriage between a man in Sibship 1 and a woman in Sibship CB. Here then is another valid exception to following the eldest to youngest rule established earlier. In the case of consanguineous marriages occurring in a kindred one often has a choice of two possible sibship lettering codes for the offspring. Thus offspring from the union referred to above could be designated with either a sibship code of 1B (derived from the father's sibship code) or CBA (derived from the mother's sibship code). The code derived from the parent who is more closely related to the propositus is the one used. In Fig. 2 the father in the consanguineous union is a sib of the propositus; the mother is a first cousin--thus in this case, the sibship code is derived from the male parent and is therefore 1B.

The names of all biologic relatives of the probandus are customarily written above the sibship line and the names of unrelated persons (spouses of relatives) are written below the sibship line. Birth and death dates are written below the symbol of each person.

Although every individual develops his own method of taking notes during interviews, there are in general two approaches to this problem:



APPENDIX FIG 3 Symbols frequently used in drawing pedigrees

The family history may be taken in pedigree form, all the pertinent information being given on the pedigree, or in outline form, the data being jotted down by sibship in the sequence in which the pedigree is assembled. Figure 1 illustrates the standard shorthand symbols used in drawing pedigrees.<sup>1</sup>

The above steps provide the basic structure of the pedigree coding system with methods for handling the common problems. From this point on, the problems become more or less specific to the clinic physician or geneticist using the system, modifications of or additions to the

<sup>1</sup> Dice Lee R. Symbols for human pedigree charts. *J Heredity* 37:11, 1946.

system may prove of benefit to meet these specific needs. It is convenient to file at least four special forms with the pedigree as follows:

1 *Case Record Sheet* The Case Record Sheet is the front piece of each kindred folder. This sheet contains the following information: genetic problem, name of *propositus*, kindred number, origin (how the kindred came to the attention of the Department), date of ascertainment, additional ascertainments and their dates, name of person who conducted the interview, and addresses of members in the kindred.

2 and 3 *Comment Sheet* and *General Information Sheets* respectively. If there is very little family history, all the information may be recorded on the Comment Sheet and the General Information Sheets may be omitted. If there is a more extensive family history, family history is recorded by sibship on the General Information Sheets and the Comment Sheet is used by the interviewer to express his opinion concerning the reliability of the information obtained from the informant and the advisability of doing further work with the family.

4 *Office Operations Sheet* The Office Operations Sheet is a progress check sheet. It contains a list of all the procedures to be carried out before the kindred folder is ready to be filed.

## SURNAME CARDS

Identification of individuals within kindreds has always presented a complexity of problems. Numerous types of name-card files have been set up with varying degrees of success. The system developed by the Department of Human Genetics, University of Michigan Medical School, has proved almost 100 per cent effective. The file is a 'surname file' in which all members of one kindred with the same surname are recorded by sibship on the same 3- by 5-in. card *Surname Cards* for the hypothetical kindred shown in Fig. 1 would appear as reproduced in Fig. 4. The surname is placed in the upper left hand corner and the kindred number in the upper right hand corner. By examining the *Public* card and comparing it with the pedigree in Fig. 1, it can be seen that each person who uses or has ever used the name 'Public' is recorded on the card (in birth order when more than one individual in a sibship is listed). Within the body of the card, the name 'Public' is abbreviated to 'P' in the cases of married females and is omitted but understood to be at the end of the names of all males and all single females. Since the *propositus* is the focal point of the kindred, her name is underlined for emphasis. If a given name or a surname is unknown, a dash may be used in its place. Thus — Wright Phillips would be the name of a woman whose given name is unknown, whose maiden name is Wright and married name is Phillips. Other examples are Mary — Phillips (maiden name unknown) and Mary Wright — (married name unknown).



|        |   |      |
|--------|---|------|
| PUBLIC |   | 4000 |
| A      | Susan John Q Joseph Janet P Williams Smith Joan |      |
| B      | Philip Michael Mary                             |      |
| C      | Mable Doe M (Mrs Michael P)                     |      |
| D      | William   |      |
| E      | Mary Jones P (Mrs William P)                    |      |

|       |  |      |
|-------|--|------|
| SMITH |  | 4000 |
| A     | Janet Public Williams S (Mrs Philip S) |      |
| M     | Margaret S Doe                         |      |
| H     | John Susan Philip Jr                   |      |

|          |   |      |
|----------|---|------|
| WILLIAMS |   | 4000 |
| A        | Janet Public W Smith (was Mrs Robert W) |      |
| H        | Peter Rosemary Paul                     |      |

|     |                                |      |
|-----|--------------------------------|------|
| DOE |                                | 4000 |
| C   | Mary Mable M Public John Jr    |      |
| F   | John                           |      |
| G   | M Mable at Smith D (Mr John D) |      |

|       |               |      |
|-------|---------------|------|
| JONES |               | 4000 |
| E     | Mary J Public |      |

APPENDIX FIG 4 Surname Cards obtained from pedigree in Fig 1

If a woman bears a child out of wedlock and the father is known this may also be indicated on the Surname Cards. For example if an unmarried woman named Mary Jones bears a child (Tom Jones) whose father is Frank Smith Mary's name would be recorded

Mary Jones (Smith) ( Mrs Frank Smith)

and Tom's name would be recorded

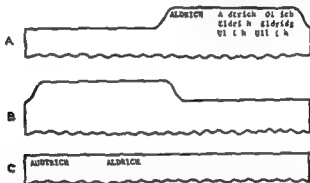
Tom (Smith) Jones

Thus a surname in parentheses is one which was never used by the individual but which at the same time has a certain amount of biologic importance, if the unused surname is unknown it may be recorded (—) with the parentheses indicating unused and the dashes indicating unknown. Quotation marks around a surname indicates that the surname is or has been in use by that individual. It may be an assumed name, a name acquired when the person was adopted, or a maternal surname the use of which is contrary to the custom in this country. Legally Tom's surname is Jones but the use of the mother's surname is a break from the

tradition of our society, in which the child has the same surname as the father, and is therefore enclosed in quotation marks

Surname Cards are filed in a phonetic alphabetic system combining the surnames which are either the same or almost the same phonetically. Thus as an example, cards for the surnames of Allman Ahlman, Ahlman Allmond, Alma, Almond, Ammon, Lmen Ullman and Ammons or of Baers, Baer, Baher, Bahers, Bahr, Bahrs, Bair, Bairs Bear, Bears Beer Beers, Bearss, and Behr would be filed as a unit, alphabetically under the most common surname in the group

As soon as one starts filing Surname Cards in a phonetic manner within an essentially alphabetic system, he must devise a system to keep those



APPENDIX FIG 5 A Guide card which precedes phonetically filed surname Cards—located in file in the alphabetic location of Ulrich B Guide card which follows phonetically filed Surname Cards C Index card which is filed alphabetically in place of Surname Cards with the surname of Audtrich Audtrich surname Cards have been filed alphabetically under Aldrich

phonetically filed cards together as a unit and at the same time make it very apparent that the cards are out of alphabetic order so they will not be used as guides for filing subsequent cards. One half-cut separator guide cards are used to separate that group from the cards filed alphabetically before and after the group. The key name (ideally the most common name in the group) determines where the group is to be filed. It is typed in upper case letters on a card with a second place cut and all other surnames to be filed within this group are typed in lower case on the same card (Fig 5A). A blank first place cut index card is then placed at the end of the group to separate it from the other cards in the file (Fig 5B).

For the names filed out of alphabetic order by being placed in one of these groups 3 by 5 in cards of a color different from the regular Surname Cards are filed in the system where those names would appear if they had been filed in alphabetic order. On these cards is typed information which will direct one to the proper place in the file (Fig 5C).

With this system in use the name of every individual in every kindred

appears in the name file with a minimum use of space. By having all relatives with the same surname on one card one can more readily determine whether a newly ascertained person is already on file. This is a very important tool in the case of more common surnames. Many Mary Smiths may be on file but the number of Mary Smiths with a brother John is much more limited.

The diagram shows five overlapping cards, each representing a different first name within the Smith family. The cards are arranged in a descending staircase pattern, showing how they would be filed together. Each card contains a header with the surname and a list of individuals with their identifiers and names.

**SUSAN SMITH**

469-CAA S S  
 1555 DB S S Philip  
 4000 H<sub>1</sub> S S  
 4938 A S Ma 1 S

**PHILIP SMITH**

156-CA P S  
 2397 B P S  
 4000 A( ) P S Sr  
 4000 H<sub>1</sub> P S Jr

**MARK SMITH**

982 D M Antho y S  
 3376-SA M S  
 4270 B M S

**JOHN SMITH**

270 A J B b S S  
 2254 A( ) J S S  
 2254 A3 J S Jr  
 4000-H<sub>1</sub> J S  
 4235 A J S

**JANEY SMITH**

1051 B Mary J S  
 1732-GB J S 2 berta  
 3689 A J S  
 4000 A J, P bll William S  
 5104 M J Mari S

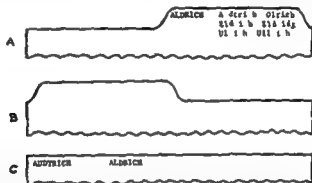
APPENDIX FIG. 6 Typical First name Cards

In the files of the Department of Human Genetics, University of Michigan Medical School, approximately 1 out of every 20 kindreds has at least one person who has or has had the surname of Smith. It is apparent that as the files increase in size a further refinement of the system is advantageous in dealing with some of the more common surnames. First name Cards are typed for the surnames that are common to at least 20 kindreds. Thus all Mary Smiths on record are entered on a Mary Smith card, all John Smiths on a John Smith card, etc. Figure 6 illustrates typical First name Cards. These cards are filed alphabetically by given name immediately after the Smith Surname Cards and are separated from the rest of the file by one-fifth cut guide cards of a different color.

tradition of our society, in which the child has the same surname as the father, and is therefore enclosed in quotation marks

Surname Cards are filed in a phonetic alphabetic system combining the surnames which are either the same or almost the same phonetically. Thus as an example, cards for the surnames of Allman, Ahlman, Alhman, Allmond, Alma, Umond, Aramon, Limen Ullman and Ammons or of Baers, Baer, Baher Bahers, Bahr, Bahrs, Bair, Bairs, Bear, Bears Beer Beers, Bearss, and Behr would be filed as a unit, alphabetically under the most common surname in the group

As soon as one starts filing Surname Cards in a phonetic manner within an essentially alphabetic system he must devise a system to keep the



APPENDIX FIG. 5. I Guide card which precedes phonetically filed Surname Cards—located in file in the alphabetic location of Aldrich II Guide card which follows phonetically filed Surname Cards C Index card which is filed alphabetically in place of Surname Cards with the surname of Audtrich Audtrich Surname Cards have been filed alphabetically under Aldrich

phonetically filed cards together as a unit and at the same time make it very apparent that the cards are out of alphabetic order so they will not be used as guides for filing subsequent cards. One half cut separator guide cards are used to separate that group from the cards filed alphabetically before and after the group. The *key name* (ideally the most common name in the group) determines where the group is to be filed, it is typed in upper case letters on a card with a second place cut and all other surnames to be filed within this group are typed in lower case on the same card (Fig. 5A). A blank first place cut index card is then placed at the end of the group to separate it from the other cards in the file (Fig. 5B).

For the names filed out of alphabetic order by being placed in one of these groups 3 by 5 in. cards of a color different from the regular Surname Cards are filed in the system where those names would appear if they had been filed in alphabetic order. On these cards is typed information which will direct one to the proper place in the file (Fig. 5C).

With this system in use the name of *every* individual in *every* kindred

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                    d d r                      e                      l f                      t                      6.0  
                    d d r                      m                      t l y d r o m                      6 9  
                    d l d o e t                      e f                      m a t i o n f 2  
                    m a                      a c d a e q u                      6.0  
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                    n h i b t                      n b y                      o r t h o t a l t                      d                      0 0  
                    d p r d i p a l y                      8 9 9 8 0 8 9 6  
                    d t e r o u d h y d                      y l t                      0 2  
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A d                      y b                      d m                      701  
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A d                      l                      d                      g                      11 y t h e n                      f 647  
A d r                      a l c o r t                      d e h y d r                      y l a t e                      o f                      653  
A d                      a l g i n d                      h                      l e n t e r o l                      y t h e a s e                      641  
A d                      a l h y p e r p l a s e                      637  
A d                      l t                      d                      a d g l                      g                      170  
                    n d l                      g l y                      g                      l 0  
A d r e n a l e c t o m y                      i n                      p e r i o d                      y a                      l y                      902  
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A d r e                      o c c u r r e n c e                      h                      r m                      e a, e                      p e r i m                      t a l                      d i a b e t e s  
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                    h                      b o o t e                      1 645 H

Suppose at some future date a young man named Mark Smith should present himself for examination. In an interview with him it is learned that he has siblings named John, Philip and Susan Smith. Smith First name Cards for these four names (Mark, John, Philip and Susan) are pulled from the files and compared to see if any of the people listed have any common kindred number and sibship coding. On the John, Philip and Susan cards it is noted that each card lists an individual belonging to kindred 4 000 who is in Sibship H<sub>1</sub> (Fig. 6) but there is no Mark Smith listed in kindred 4 000 H<sub>1</sub>. However with this amount of positive correlation, the information obtained from Mark and that contained in kindred 4 000 should be compared—birth dates, other names and addresses are all of help in determining whether or not the kindreds are one and the same. In this example there were three positive correlations. However, in attempting to trace relationships it is sometimes helpful to compare records with as few as two positive correlations. If Mark had supplied no information about siblings but did state that his mother's name was Janet Public Smith, a card in the file with Janet — Smith (woman with given name of Janet, maiden name unknown, married name of Smith) would supply one positive correlation. A card in the file with the name "— Public Smith" (a woman whose given name is unknown, maiden name is Public and married name is Smith) would also supply one positive correlation. Thus the location of the name Janet Public Smith in the file (Fig. 6) would supply the two positive correlations required for further checking.

## DIAGNOSIS CATALOGUE

With the increase in number of records on file a Diagnosis Catalogue becomes a helpful tool for locating all kindred records relating to a particular disease. This catalogue could be of loose leaf notebook or card type depending on how much information is to be recorded about each kindred. Each disease is recorded on a separate sheet or card; these are kept in alphabetic order. Since many clinical entities may be referred to by more than one name (imperforate anus vs. atresia ani) or may be alphabetized under more than one word (Patellar nail or Nail patellar syndrome) a list of the synonyms of diseases already on file should be kept so that any disease apparently not yet in the catalogue may be checked against this list. It has proved helpful to have the following information on each diagnosis sheet: trait kindred number, kindred name (surname of the propositus or in the case of a married female, maiden surname plus married surname), year of examination, type of inheritance, number examined and affected with the disease, origin and remarks.

Allu na ker toe n 432  
 huk get ll 444  
 local d 430 435  
 ung e h ed alh az 445  
 g t e f 444  
 m tab h d f t 445  
 mammals 431  
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 Alb etym lgy f 428  
 Alb d m ( Alb m m l b d)  
 Alb m l m l ( Alb m a B ly  
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 hamu try f 1380  
 distr b u f 1381  
 f t ty d t asp t by 1387  
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 Alb m B naly 1380 1384  
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 lect ph t p tte 1383  
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 Al pto a d 1384 1385  
 arthrit 397 403  
 d E 404  
 yai 404  
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 l m cal f t a 397 E  
 d teet f heter m tes 418  
 d m t f m f 418  
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 m f m placet ac t and hyd l a 410  
 b m ge t m d s u d a s 410  
 p-hydr yph s lpyru d s u d a s 410

Al pt m l m l y la et t o d  
 m 410  
 ty t sam se 410  
 m t b l a d f t 409  
 yoca dial uf t m 40  
 t gm t t a n f t l a g 397  
 p t t t s 405  
 l t 405  
 pt d t t b l d l k 405  
 d 417  
 te ts f 398  
 t t t f 419  
 d ty a 384  
 e b a 397  
 t m C d f y 416  
 y b g 398  
 Al h l a m d p o p h y 908  
 Ald byd s u d a s 7 455  
 (S l a a n h e d e e)  
 Al l 91  
 m l a f m l y d y 70  
 d g e e t y t h y t e 1045  
 Aldost rone m d y n a m s p u o d f e d t a r  
 9 3  
 f m t n f 447  
 ACTH d 653  
 p e d p t y 899 891 890 901  
 alt b l d o s u l 69  
 Aldost m p r i m y 903  
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 Al l p h o s p h a s e b y l l m l b t f  
 1372  
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 E o f m t t a 37  
 F m y a d m l 2  
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 t b l a d o s l 57  
 t m D- t t k t a 1178 1183 1212  
 Al t d 7 0  
 Al t o s r e t m f r e d t 727  
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 Al p a e-3 17 a d t l 150  
 Al d b t l l 74  
 Al d b t e p l m b l 1346  
 Al d b e t t N m h y l t m d  
 t a 1346  
 Al l p r o p l a e t y l e b m l ( h e d d)  
 Al p c e l l a g l a 171  
 A t f m l y d o c y f l 489 503  
 g f a n t m  
 ald l a s e t a t y 5 0  
 t m h g e s n 564 E  
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 l p d a n 567  
 t d a 508  
 t a l y t m 488  
 h r y r e d p o t 554  
 l m a l f t r e s o f 554 E  
 d f i n t a o f 554  
 d a n y h m a t 556 509  
 p l p t a f m e s n 556  
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- +Anon l vulans ac d d hyd ae Sw d h  
 p phy 984  
 +Amo ph ylg comd m d b oay then  
 f 240  
 hyp bulruh em ts 244  
 Amo pyn dgl m d ad t p th  
 y 130  
 Amm ni t b l r: ret d y tles f  
 1243  
 nsef mgl t m 1 6  
 Amm mgly s l t p i p ly  
 8 6 886 891  
 Amm m m k t gen: f t f 408  
 AMP 3 ( Ad yl m ac d)  
 Amjlae ae m esse talf mual hyper  
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 m y l p e e t o e )  
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 B t f F S H G u O  
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 A d o e t 317-d m t t  
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 f r o m 17 h y d s p g e m t 648  
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 h y p t n s d g t l y i m f r  
 A m f m l a l m o c y t ( o e T h l a s s )  
 h m l y t ( l l m l y t )  
 A m h y p o c h m d b l a s t h e d  
 1132  
 A m m d l k a e m f t ( T l l a s s m )  
 A y m u t l a s 140  
 ( S e e d A t l a s )  
 N N a h y d r f m l t t h y d f l d  
 p r s t h 696  
 A n d n s s  
 A t e c d d b e t H  
 A t h o c y n s h r t f 5  
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 A t h u l d g l a d h y p e b l  
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 A t s p t d b e t r e 71  
 A t d t h m ( V a s p a s )  
 A t t m p h l e g l b l 1149  
 a s s y f 1167  
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 A n t l p h l g l b h d f y ( H m o -  
 p l l )  
 A t p s 1033  
 A p l a s t m h r e d t r y p h o c y t o e 1010  
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 A b 124  
 A b t l p e t o e 10  
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 A g u f e d b k t l f b a y b 787  
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 t t f 1313  
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 g t y 680  
 t 683 688  
 l m 494 689  
 f e e t 685  
 h d s 685  
 d l o o s 406  
 s m 690  
 t m t f 735  
 A s t t m 12  
 A s t e a c l i d l t f t f A C T H  
 60  
 d f y f g l y m t b l m 198  
 g l d p t h y 94 18  
 d h m g t l 398  
 d b p t 841  
 m t b l m f 488  
 l t p m 400  
 r y 458  
 A b d d t h y d d t t 81  
 A s p t t b m y l t f a s h b t b y  
 y t d j l d 787  
 A T 10 1188  
 ( S i D h y d t h y t l )  
 A t b r m l t g o e h o e 40  
 A t l m l l p t 40  
 t l f l l p l l t l 4  
 d t h m t o s 400  
 A T P ( d o e m t r i c e p t t ) g l s l 92  
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 B d t y p h o c y t 1024  
 p r o t y t h e s 34  
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 A T P a s e 166  
 A t o e 0  
 2 A d m d t h d a s 708  
 2 A h y p t h a d a n t h d a s 768  
 A a s d p r s t h e s 00  
 d d s t 40  
 A t m h y p o p h o s p h a s e 1374  
 A l f a d 1033

# 1428 THE METABOLIC BASIS OF INHERITED DISEASE

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 h t ry of 588  
 hyp ra u la 506  
 Janaky B lack u ky f -4  
 n Jewa 5 572  
 juv left of 575  
 r als d e f 3  
 Kuf f m 54  
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 b n b d l a etab l f 4738  
 x dat ed amon f 474  
 t s to f 474  
 A o ed ad y l t s pr t y 12 34  
 A no a d o daee and thyr l ad t t n  
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 = n a id daee 1407  
 t amino ac fo daee 1407  
 nd b ched h a o ed 474  
 A o d t nspo t n as p m t t  
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 A o cyl plosph t f t n g t syn  
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 A o d br b d ba m  
 y t os 1409  
 d y t n 1313  
 n gal toe m 47 210  
 m ples p m daee 478  
 v m n D ros t t k t 1187 1192  
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 y Amu b tyr a d 795  
 4 Amu o-5- md l c th m l d  
 fo matio 715  
 i a th n boey th 68  
 5-Amu m da lo-4- e th m d m l t l  
 n pu ynth 695  
 5-Amu m da ol -4- e b y l b lo-  
 t de n p r ynth 643  
 5-A o mid l b bul t d p 3n  
 thea 694  
 5-A noe id lo-4-(N- s y l arb d )  
 bonu l t de n p y t 695  
 p Am a s U tyr d n body Bu de 793  
 d r t n f 795  
 f m t on te 797  
 glut met ane am 794  
 p-Am m tyra d l gh cret ra of 792  
 met bolom of 793 793  
 plasma a d r a l l f 707  
 renal tubul e absorptio of 797  
 at i m d r t 795  
 and thy 793  
 a d tal n 794  
 p-Am sub tyr a d a 14 7928  
 age sex d a t s so ne t and  
 800  
 b mod l ty f 799  
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 ch m t g phylo 789  
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 dihyd ty r n fac l g nd 791 797  
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 im l r d aee 732  
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 im l l gl 792  
 m l m th y t o s t e d s g 794  
 im m g l n d ocy 793  
 after ut m i ad t 793  
 q ut u e r l t n s f 98  
 rac l a t 803  
 r a l h u n 90  
 aft RNA feed g 794  
 and th y m d e f ed ng 797  
 and th y r feed g 797  
 l t b e uloe a 792  
 d p- d b t y d feed ng 797  
 i l o m t g phy 792  
 after y ad t 793  
 i y g h l d 800  
 a Am o-6- k t d t o a c d ALA y th a  
 90  
 po p l y m l o s y d 948  
 f m c y l C A a d gly 90  
 a-Am l t l m a c d 947  
 in b l 960  
 lo y n t h e s f 9495  
 nd a t o of 951  
 eo r a t g p l l k g 985  
 = m phy 982  
 y th o c y t p t t n by 960  
 m l d p m t g 961  
 met b l m n 969  
 a d p h t o e t y 962  
 in Sed m d p aom g 942 960  
 f B w d h p p l y m 984  
 ynth nh b t f 900  
 urina y et of 900  
 f S uth Afr b p l y 992  
 p-Am n l v hno d d h y d a s p p in  
 951  
 inh b t o 951  
 m P D G ynt 960  
 p t e o b y g l t h m e 951  
 req a t f o 91  
 sou f 951

B ed ch betes ( H m hrom toni )  
 B f m r h m g t d dasei 412  
 B g M H 1274  
 B it C H 1177  
 B ty yl C A l f tly day th is 96

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C d n 1306  
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 C AlR ( 3-Am mod 1-4-carb yb ac d  
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 b m 566 548 613  
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 n G her ocl 605  
 H oae 611 619  
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 t port f t  
 t h u f 616 H  
 Cerebromd l p d m (or H H s disease)

## B

- Bacillus acidophilus* H O prod ct 1407  
*Bacillus subtilis* H O prod to 1407  
 Bact ph g  $\phi$  114 DNA n 7  
 BAIB (  $\beta$  Amylase b t y n acid)  
 Barn, J mea A 776  
 BAL (Brit ha t l w t) 16 8 8  
     Wilson's disease 16 8 8  
 B b t a l d g l u o m a d o d t p t h  
     way 130  
 B b t t r C 327 1367  
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     thy odism 9 8  
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 Badle G W 5  
 B n A l e a d r G 409  
 B h n o a c d d 559  
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     s u f a o n t c i n 1180  
     t h e o r y o f 1147  
     t u m e m e s s m t f 1164  
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     1164f  
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     c l i n i c a l a p p r m e s 1181  
     d m o s t u t e t a f o 1168  
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     d f e c y 1153  
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 C N 1110 1033  
 C g u l t i a c t r a ( Blood-cl t u g f a c t r a )  
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 C r p d 8 ( C r t l )  
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 C g t a l h m o l y t a j d 1015
- C g n t a l u h l y t e j a d u ( C n  
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 C u J m a W 557 519  
 C n a t t t a l h b t u d j f t u n 2448  
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 C r t o l 659  
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D hyd ocp nd oate h ype t nou adre  
 g tal y d 60  
 n f 648

D j l t f l y d ocy 70  
 reb oad n of 9  
 ph nlyk t 336  
 ptang my l a r 9

21 D y m d d h t l y d m 8 6  
 Dec y rt oate n d p e ad p l y 309  
 870 880

11 Dec y t oot ( or C te ne)  
 1 D y t 4 600

21 Dec y t t met b hite f 660

11 D y 17 hydr s t te ( C t o-  
 l )

D y b f as 4  
 l d II d l d 01

D y b l d (DNA) 8 o-  
 b t y n ac d r 798  
 b t ph g 3  
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 d g f 31

II y bos l ph ph t f p l a-  
 de 01

II y thosa th pre re 766

D f bosph phosphorylase 86 161 621

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 adrenal ued p od m 61  
 h 173

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 t 81

De moete f 541 644

D T F ul y d ( F u s )  
 d m )

Do t lock E m s d 1.32

Diabetes ins p d a h edia y pat tary 1389  
 epl rog uo ( as pema rem t t bel w)

D bet m d p m ut t 13 12747  
 h al po te 1284  
 l m na 1 85  
 d h y d t n 1 85  
 d g os f 1389  
 t re l g t o f 1 88  
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- Cortisol adrenally 657  
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- Δ-Cortisol adrenally 655  
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 C y t o a m o a d n 1309  
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 a m u k 1316  
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 a m e 130 1321  
 r e l f a t a 1316 1317  
 r l t b l t r a p t 12  
 t a t a f 1305  
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 y t 130 13 2  
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- C y t d i p h o s p h a t e h k p t h a y 517 90  
 P C a n d 300  
 t a f a s 594  
 C y t d p h o s p h a t e s n g l y p h o s p h a t d  
 y n t h 517  
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   thy d 931  
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   d p e d h y m thy d 931  
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